CHORIO-ALLANTOIC GRAFTS OF FRAGMENTS OF THE TWO-DAY CHICK, WITH SPECIAL REFERENCE TO THE DEVELOPMENT OF THE LIMBS, INTESTINE, AND SKIN

by

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In a previous paper (Murray, 1926) experiments were described in which limb anlagen of the two-day chick, together with certain other tissues, notably somites, were grafted upon the chorio-allantois of eight-day chicks. After life as a graft for eight or nine days, it was found that these anlagen had developed jointed and branched cartilaginous structures, which, while very far from being normal limbs, were still obviously recognizable as attempts on the part of the graft to form limbs. Owing to the fact that these grafts (Nos. 64–66 of the previous paper) contained, in addition to the limb anlagen, overlying ectoderm, underlying endoderm, vertebral plate tissues as yet not segmented into somites, and doubtless parts of the lateral plate not concerned in limb-formation, it was not possible to claim that complete self-differentiation of the limbs had occurred. Any of the tissues present might have been necessary for the further development of the limb-anlagen. In 1898 Byrnes found that the normal development of the limbs in Amblystoma and Rana is independent of the presence of the muscle-plates of the limb region; Lewis (1910) confirmed this for Amblystoma, and was himself confirmed by Detwiler (1918). The latter found that the limb anlagen of Amblystoma are already determined (to be limbs: we are not discussing the determination of axes of symmetry) at the stage of open medullary folds, and can be transplanted. Harrison (1918) found that the mesodermal component of the limb anlage is the active partner, while overlying ectoderm is not specific, and can be replaced by ectoderm derived from a region where limbs are not formed without preventing the development of the latter. It would thus seem improbable that either somites or the ectoderm of the region would be necessary for the development of the limbs of the chick, but, considering the surprising differences seen between even closely related species in points of developmental physiology, it remained necessary to test the matter in the chick

so far as technical difficulties permit. This has been done for the somites by making grafts similar to those described in 1926, but containing no vertebral plate tissues. With reference to the possible importance of the endoderm in limb determination, it should be stated that Marx (1926) found that a piece of the archenteron of Triton, placed in the blastocoel of another embryo at the onset of gastrulation, induced the formation of a secondary medullary plate in the overlying ectoderm. The only way of testing whether the endoderm plays any part in limb determination in the chick would be to graft the limb anlagen of two-day chicks in the absence of underlying endoderm, and this has not yet been done. It is worthy of mention, however, that whereas in Marx's experiment there were no other tissues lying between the engrafted piece of archenteron and the wall of the blastocoel which formed the secondary medullary plate, in the case of the chick the endoderm is, *at the time of grafting*, separated from the limb-forming somatopleur by the splanchnopleur and the coelome. It thus seems unlikely that the endoderm, if it is an "organizer" for limbs, can exert its function after grafting: that is, the limb anlage probably develops independently of the endoderm from the time of grafting onwards, whatever action the latter may have had at earlier stages. This is clearly very little more than mere speculation, and we have really absolutely no knowledge of the mechanism of limb determination in the chick or in any other animal, with the somewhat doubtful exception of the Amphibia.

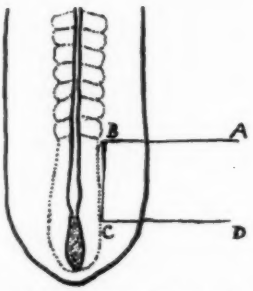
The object of the present paper, then, was to test the dependency of the two-day limb anlagen for their development upon the tissues of the vertebral plate, beginning when the vertebral plate in the limb region concerned is not yet segmented into somites. The information relating to the intestine and the dermis was obtained quite incidentally.

MATERIAL AND METHODS.

The chicks from which the grafts were taken had been incubated for two days. From the available "donors" individuals were selected in which somites had not yet developed opposite the region in which the posterior limb-bud later appears. The anterior limb develops opposite somites 17–19 and the posterior limb opposite somites 26–32 (Lillie, 1908, p. 184). The grafts were prepared by cutting out of the donor a small, roughly rectangular piece, whose anterior border lay posterior to the level of the last somite formed, while the medial border lay just lateral to the vertebral plate, and the posterior border anterior to the level of the anterior end of the relic of the primitive streak. The lateral border of the piece lay at various distances from the mid-line of the chick in the area pellucida. The nature of the graft is indicated by ABCD in text figure 1.

The method of grafting was similar to that described in the previous paper,

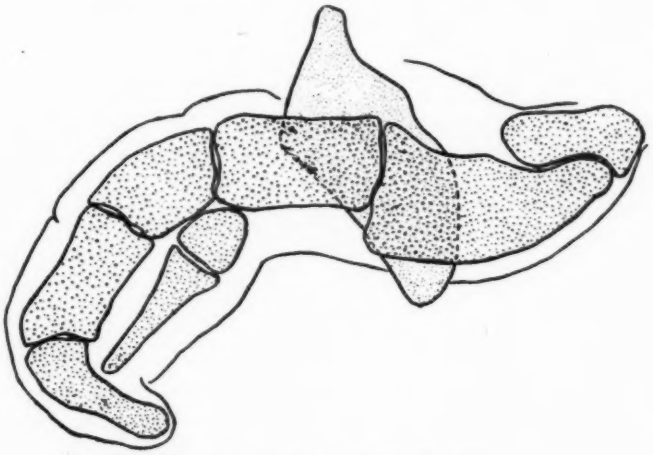
the grafts being placed upon the chorio-allantois of an eight-day chick, and allowed to remain as grafts for nine days. On removal from the host the grafts were fixed in Carnoy's fluid, stained in methylene blue, and cleared by the Spalteholz method. They were then studied, and in some cases subsequently sectioned.



Text figure (1). The posterior end of a chick of 27 somites (after Lillie, modified). × 20. The region taken from the donors and used as grafts is indicated by the quadrilateral ABCD.

The Grafts.

It will be simplest to consider the results in the following order: (1) the limb anlagen; (2) the endoderm and splanchnopleur; (3) the ectoderm and the remainder of the somatopleur. The general anatomy of the grafts will be described in connection with the behaviour of the limb anlagen.



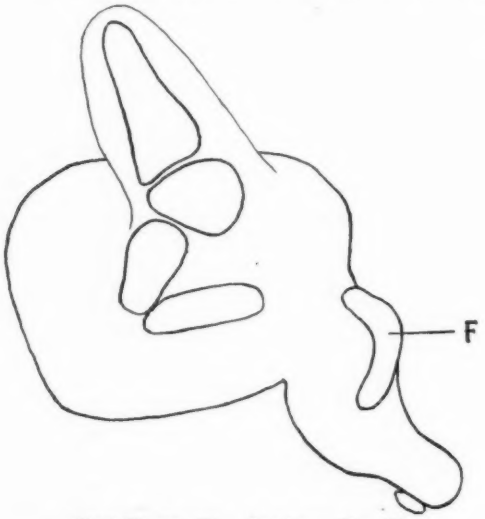
Text figure (2). Specimen No. (1).

(1) *The Behaviour of the Limb Anlagen and the General Anatomy of the Grafts.*

No. 1. Text figure 2.

Origin. From a chick of 25 or 26 somites. Since this graft, like the others, was taken from behind the region in which somites were differentiated, and since tissues opposite somite 26 are involved in limb-formation, it is evident that this graft did not contain the entire anlage of the posterior limb. It is possible that but for this fact it might have shown a closer resemblance to a complete limb, but as we do not know whether the limb anlage is at this stage a mosaic or not, we cannot be sure upon this point.

Graft. This graft was studied after clearing, but as I wished to keep the specimen, it was not sectioned. As the figure shows, it consisted of a main rod of cartilage in six segments, while a two-segmented branch comes off from the third segment of the main rod. There is also a flat plate of cartilage lying beneath the main rod, which is suggestive of a piece of pelvic girdle.



Text Figure (3). Specimen No. (2). F is a fold of the edge of the flat plate, not a separate element.

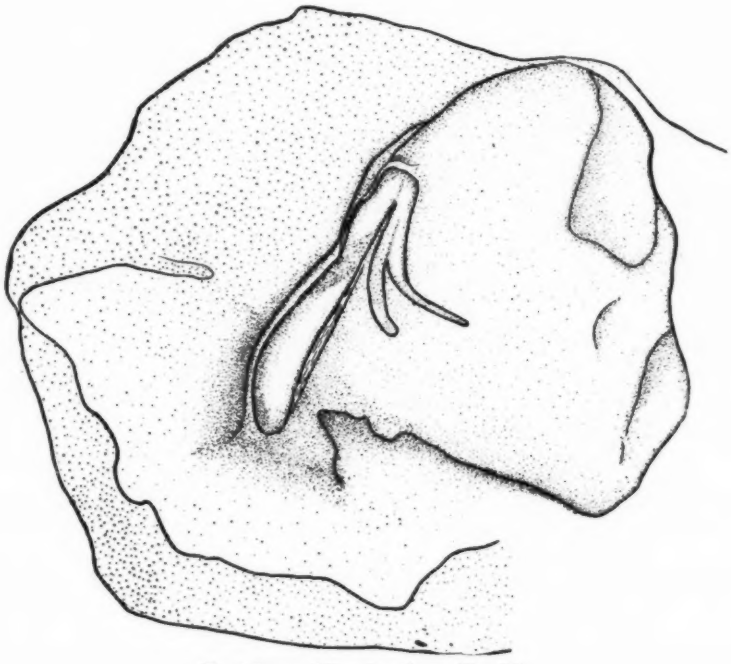
No. 2. Text figure 3.

Origin. From a chick of 23 somites.

Graft. The graft consists of a roughly rectangular, flattish plate of cartilage, from which a "tail" projects at one end. Arising from this flat plate is a structure which appears to be a digit with four phalanges, the terminal phalanx having a form quite closely approaching the typical form for terminal phalanges. There is also a small nodule of cartilage at one end of the "tail."

This specimen was studied both from the entire cleared specimen and from sections.

The latter showed a slight error to exist in the figure. The second and third phalanges of the "digit," which are drawn in the figure as nearly touching, were really separated by a considerable space. The figure was drawn from the entire preparation after clearing. The sections also revealed the presence of a considerable area of skin with a dermis and well-developed feather germs.



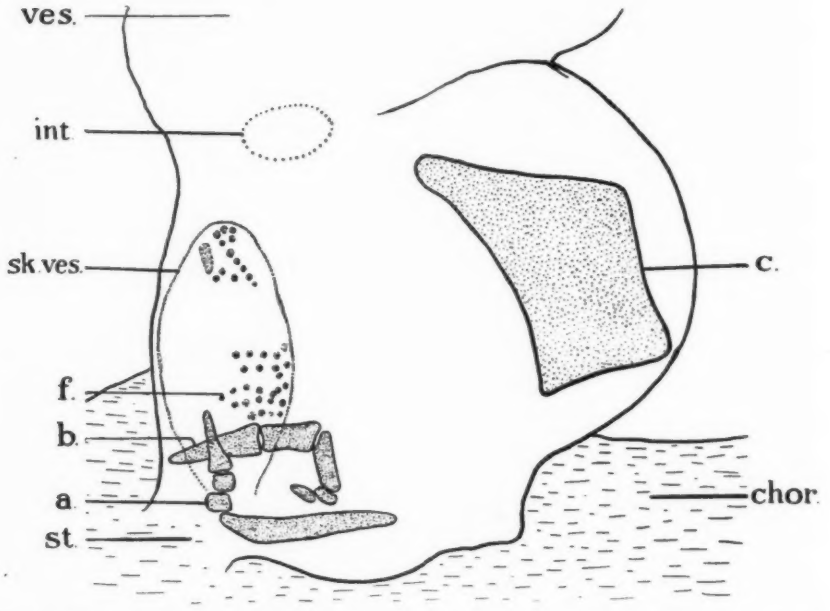
Text figure (4). Specimen No. (3).

No. 3. Text figure 4.

Origin. From a chick of 25 somites.

Graft. In this case, as in others to be described, the skeleton of the graft developed in the wall of a curious large vesicle, which was hollow for the most part, and which was attached to the endodermal side of the chorio-allantois by a short stalk. Upon removal from the host this vesicle was large and rounded, but became considerably shrunken during the preparation of the specimen, the distal end becoming collapsed and folded, as indicated in the figure.

The cartilaginous part of the graft consists of a bar of cartilage, from which arise two more slender rods, apparently jointed to the main bar. The main bar appeared to be encircled by a thin layer of bone. The specimen was studied in the entire preparation only.



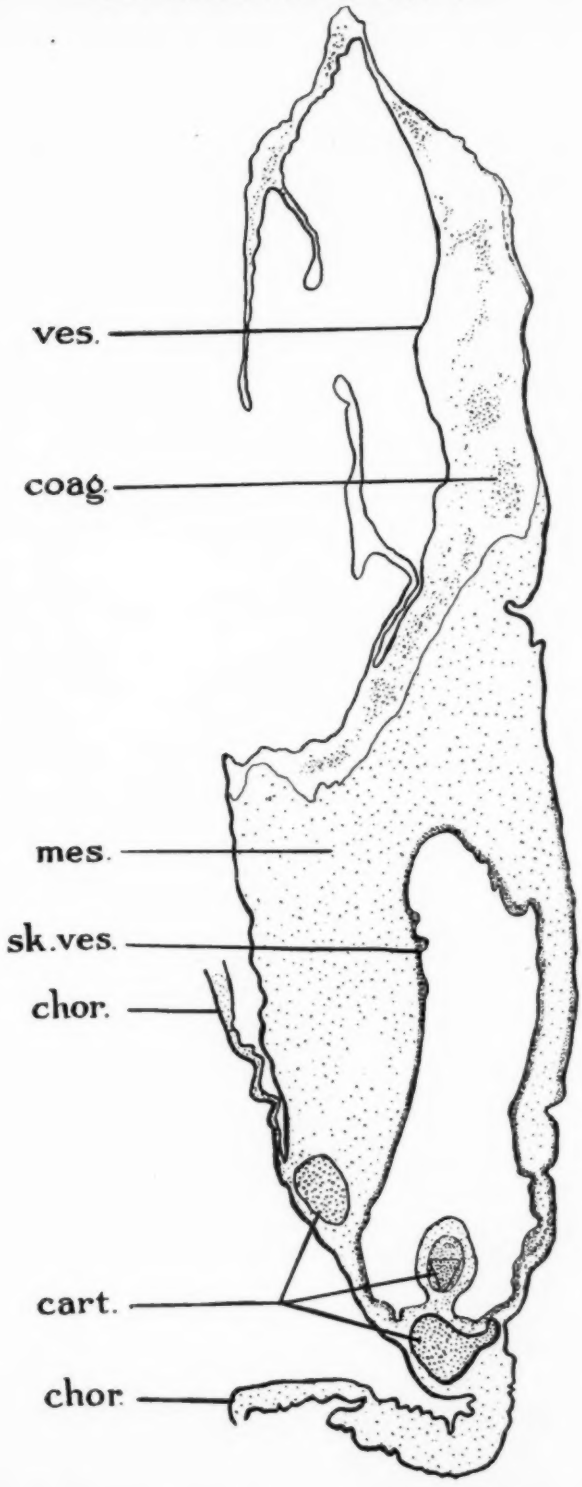
Text figure (5). Specimen No. (4). The upper part of the vesicle is not shown. a, b, c, cartilages; chor., chorio-allantois; f., feathers; int., intestine; sk.ves., skin vesicle; st., stalk; ves., vesicle.

No. 4. Text figures 5 and 6.

Origin. From a chick of 26–27 somites.

Graft. Here again the cartilaginous part of the graft developed in the wall of a large vesicle (ves), which was attached to the main membrane by a narrow stalk. This vesicle is hollow, and is partly divided into two chambers by a shelf of tissue in which lies a fragment of intestine, to be described later. The wall of this and similar vesicles of other specimens is for the most part very thin, but becoming thicker below, as it approaches the region where it joins the main mass of the nodule. On its inner and outer aspects the wall is bounded by a thin epithelium of flattened cells, between which lies a quantity of mesenchyme,

Text figure (6). Section through specimen No. (4), vertical to the chorio-allantois. Cart., cartilage; chor., chorio-allantois; coag., coagulum in vesicle; mes., general mesenchyme of nodule; sk.ves., skin vesicle; ves., vesicle.


ves.
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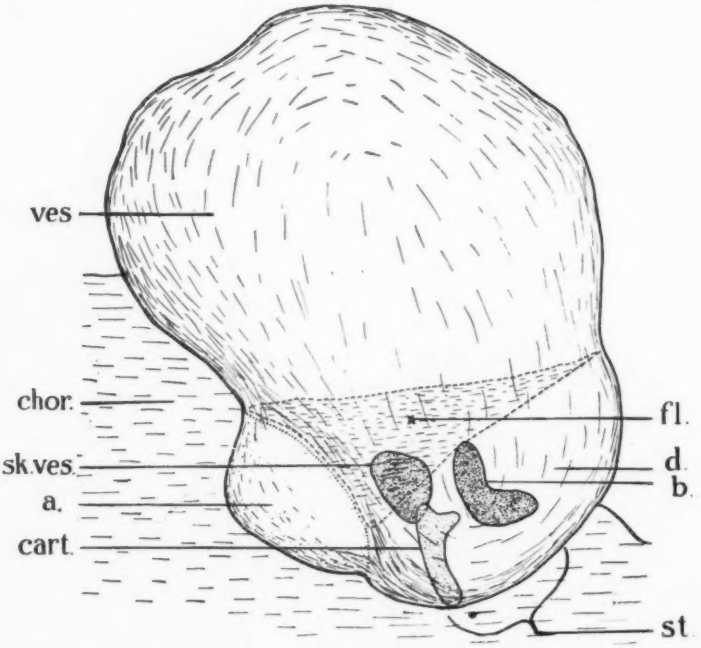
which is quite considerable in the thicker regions, very exiguous or absent in the upper, thinner parts. Running in this mesenchyme are a few small blood vessels. The contents of the vesicles were only examined after fixation, and then proved to be fluid and to contain varying quantities of an amorphous precipitate or coagulum (coag.). I have no idea what the nature of this material may be. It stains in eosin. The cartilages of this graft consist of two jointed rods (a and b, figure 5), and a flat plate (c, figure 5). Reference to the figure will show the



Text figure (7). Specimen No. (5). a and d, more solid regions in base of nodule; b, gut. cart., cartilage; chor., chorio-allantois; fl., floor of vesicle; sk., skin vesicle; st., indicates position of stalk of vesicle; ves., vesicle.

arrangement of these better than any description. It will be noticed that the flat plate of cartilage was some distance away from the two jointed rods. The jointed rod (b) lies partly beneath (sk. ves.), which is a hollow space in the wall in the nodule. The histology of the wall of this cavity shows it to be skin, and so evidently derived from the graft, and having a definite dermis. Projecting from the wall of this vesicle into the lumen of the cavity are many young feathers (f). The other jointed rod (a) projects at one end into this vesicle.

Occupying approximately the position indicated by the dotted circle (int.) is the fragment of intestine to be described later.

Plate 1. Figure 1.

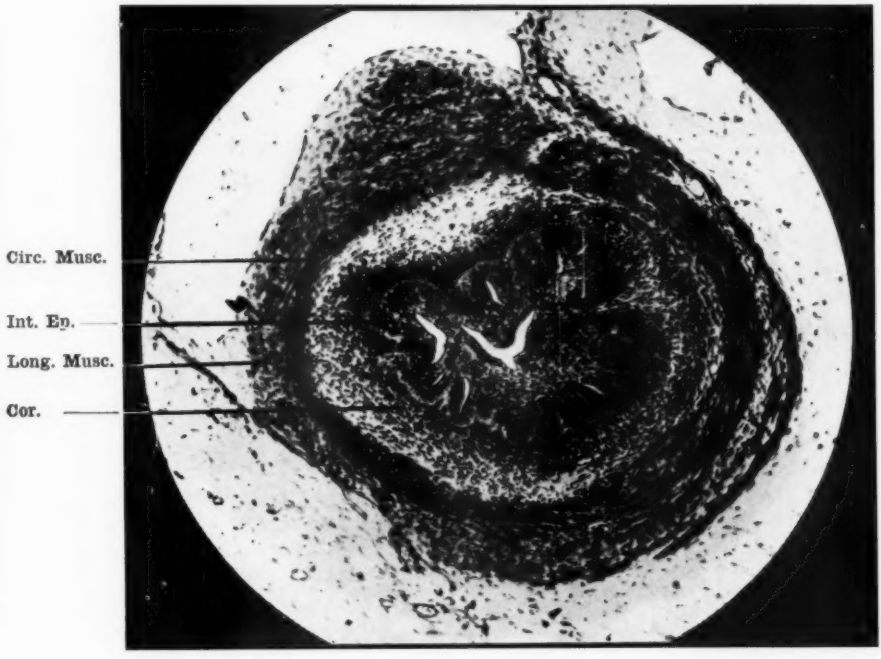


Plate 1. Figure 2.

Plate 1, Fig. 1. Intestine of No. 4. × 110 [110]
Fig. 2. Intestine of No. 5. × 161 [141]

Figures in [brackets] are magnifications after reduction.

NOTATION.

Circ. musc., circular muscle; cor., corium; int. ep., intestinal epithelium; long musc., longitudinal muscle.

No. 5. Text figure 7.

Origin. From a chick of 21–22 somites (left side).

Graft. Again the graft has developed in the wall of a large vesicle. The horizontally shaded area marked (fl.) is the floor of the hollow portion of this vesicle, while (a) and (d) represent the more solid regions in the base. Embedded in these are the various parts of the graft. The recognizable parts of the graft are in three masses. There is only a single bar of cartilage (cart.) of the form indicated. Lying to the right of it is a curved fragment of intestine (b), and above it and slightly to the left is a vesicle (sk. ves.), whose wall consists partly of skin with a dermis, and having feather germs.

(2) *The Behaviour of the Endoderm and Splanchnopleur.*

Since each graft consisted of a small rectangle taken from the side of the donor, it is clear that there was involved in the graft a small piece of endoderm and of the overlying splanchnopleur. Their subsequent behaviour as grafts is of interest.

In three out of the four grafts which were sectioned there was found a short length of intestine, in two cases tubular and in one case solid.

The Intestine of No. 4. Text figure 5 (int.), and plate 1, figure 1.

The gut is a somewhat irregular tube, bent on itself at approximately 90 degrees, and occupying roughly the position indicated in the text figure. It has a definite lumen, epithelium, and muscle layers. The epithelium is tall and columnar, with few or no goblet cells. Beneath the epithelium is a corium of connective tissue, which is very densely cellular beneath the epithelium, less dense beneath the muscle layers. There is a well-defined layer of circular muscles, and outside this a somewhat thinner coat of longitudinal fibres. The lumen is closed at both ends, which are covered externally by epithelium and an irregular knot of muscle fibres. For the most part the structure lies embedded more or less deeply in the mesenchyme of the chorio-allantois, a loose and probably syncytial network. In part it lies in a kind of shelf of tissue across the lower end of the space enclosed inside the large, hollow vesicle, which is such a conspicuous feature of the intact graft. Where the fragment of intestine abuts upon this cavity it is covered on its exposed face by a little mesenchyme and by the flat pavement epithelium which lines the cavity, but on the face which is turned towards the general mesenchyme of the chorio-allantois, and over the entire surface when it is wholly embedded in the latter, there is no epithelium of any kind separating the outer muscle layers from the mesenchyme. In other words, the peritoneum has not developed.

A long, narrow diverticulum, having an epithelium like that lining the lumen of the gut, runs off from the latter, and, accompanied by a few strands of longitudinal muscle, can be traced through a great many sections, finally ending blindly. Its significance, if any, is unknown.

The Intestine of No. 5. Text figure 7 (b), plate 1, figure 2.

This piece of intestine on the whole resembles the last described, but there are certain differences. The structure lies mainly in solid mesenchyme, but projects at one end into an internal chamber lined by the same type of flat epithelium as was described in the last case, and again, as in No. 4, this chamber is a continuation of the cavity of the large vesicle. Like the last, this intestine has no peritoneal covering, but is covered by the flat epithelium of the cavity where it projects into the latter. It has a definite but much folded lumen, a corium which is more dense under the epithelium than under the muscle layers, while there is again an internal coating of circular and an outer of longitudinal fibres, the latter being less definite than the former.

The principal feature of difference from the last described specimen is in the much greater folding of the epithelium, which in other respects resembles the former. I am inclined to the view that this folding may be interpreted as an irregular attempt to form villi.

The Intestine of No. 6. Plate 2, figure 1.

This specimen, since it proved to contain no cartilaginous structures at all, was not described in the previous section. There is a small oval vesicle with a narrow neck projecting from the endodermal surface of the chorio-allantois, and near the base of this, in the chorio-allantois itself, lies a most extraordinary structure, which can only be interpreted as a fragment of intestine turned inside out.

This is in the form of a large, finger-like body, lying for the most part free in an internal cavity in the chorio-allantois, but little bigger than itself. It is roughly elongate-oval in shape, and at its base it is attached to one end of the cavity. Histologically it has a core of longitudinal muscle (longitudinal with reference to the length of the whole body); this is surrounded by a coat of circular muscle, while outside this again is a corium, which is densest beneath the covering epithelium. The latter closely resembles the epithelium lining the lumen of the intestine in the other two specimens, that is, it is columnar in form, while its cells are of a type similar to those found in the other intestinal epithelia. This epithelium covers the finger-like projection, and at the base of the latter is reflected back to form the inner lining of the space. The columnar form, however, is not retained by the epithelium lining the space, except for quite a short distance near the base of the intestinal projection, and even in this short distance the cells become progressively lower and lower, until they become flat and pavement-like, resembling those lining the spaces into which the two previously described fragments of intestine project. It differs, however, from these other specimens in that the space in this case is not in any way connected with the cavity of the big vesicle.

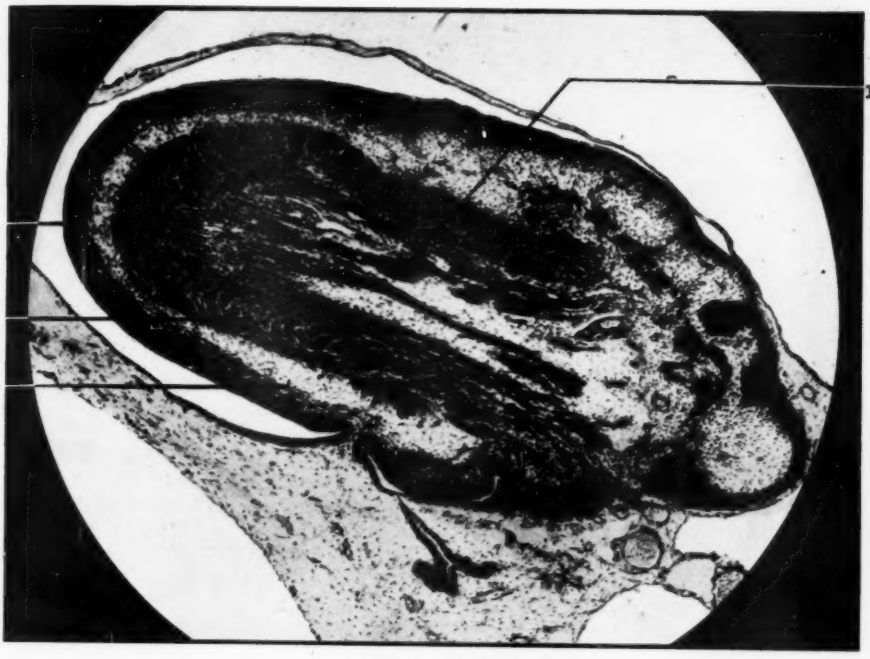


Plate 2. Figure 1.

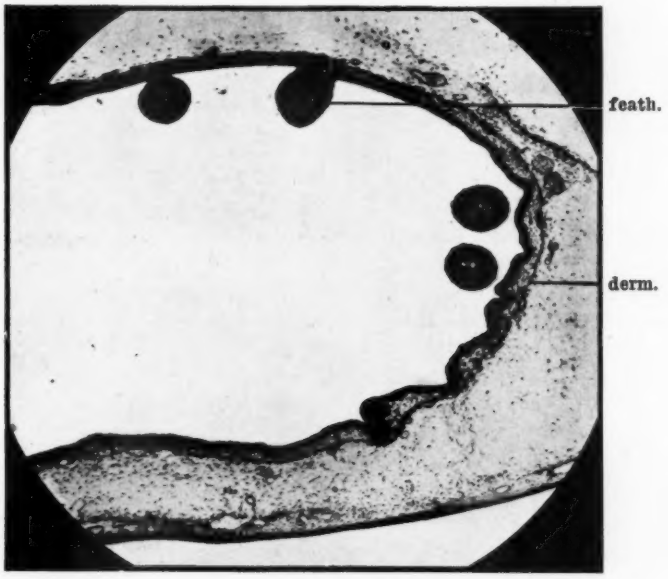


Plate 2. Figure 2.

Plate 2, Fig. 1. Intestine of No. 6. × 72 [72]
Fig. 2. Skin vesicle of No. 4. × 83 [66]

Figures in [brackets] are magnifications after reduction.

NOTATION.

Circ. musc., circular muscle; cor., corium; derm., dermis; feath., feathers; int. ep., intestinal epithelium; long. musc., longitudinal muscle.

On first examination I imagined this specimen to be a length of intestine, in which the epithelium, which I naturally expected to find lining an internal lumen, had for some reason degenerated, but the difficulty of this intrepretation became apparent when I remembered that in birds, as in mammals, the inner muscular coat of the intestine is circular and the outer longitudinal, whereas on this hypothesis these relations would be reversed in the graft. This suggested that the specimen was possibly a piece of intestine turned inside out, in which case the covering epithelium should resemble that lining the lumen of the other specimens, and this I found to be the case. That this is the correct view is further indicated by the fact that columnar epithelia of this type are found nowhere in the grafts other than as linings of intestinal tubes. The view I have adopted is consistent with the following facts: (a) the epithelium lining the external face of the intestine in this case almost exactly resembles that lining the lumen in the other two specimens, and such epithelia are not found in the grafts except as lining the lumina of intestines; (b) the muscular layers are present in the order expected if the hypothesis be true, but in the order opposite to that expected if the real position of the intestinal epithelium is in the centre of the body and if it has degenerated; (c) the facts in this case are not inconsistent with a hypothesis to be advanced (see discussion) to account for the other two intestinal anlagen having formed tubes.

Histologically, the epithelium lining the face of this intestine differs from that lining the lumen of the others only in the presence of certain gland-like structures, which I have not been able to find in the other epithelia, and in the absence of folding. The latter point will find its explanation in the discussion.

The gland-like structures consist of small groups of cells sunk below the general level of the epithelium, and apparently enclosing a lumen between their distal ends. This lumen appears to communicate with the exterior.

It may also be pointed out that, since the epithelium is reflected over the internal face of the cavity in which the intestine lies, the latter is not completely covered by the epithelium, the basal part where it is attached to the wall of the space having thus no epithelial covering, the mesenchyme lying among the muscle fibres, being thus in direct communication with the general mesenchyme of the chorio-allantois.

(3) *The Behaviour of the Ectoderm and Adjacent Somatopleur.*

It has been mentioned that three of the sectioned grafts proved to contain areas of definite skin with young feathers.

The Skin of No. 4. Text figures 5 and 6 (sk. ves.), plate 2, figure 2.

The skin is found lining the wall of a roughly oval-shaped cavity, completely closed, in the position indicated in the text figures. Its histology is quite typical,

having an epithelium consisting of a layer of columnar cells covered with one or two layers of flattened cells. Beneath this epithelium is a definite and clear dermis (plate 2, figure 2, derm.), consisting in the main of a densely fibrous connective tissue, whose fibres take a wavy course beneath the epithelium, while in other areas the fibrous component becomes less in amount and the dermis is more densely cellular. This histology alone would leave little doubt possible as to the nature of the structure, but final proof is presented by the development of a considerable number of young feathers (feath.), whose form appears to be perfectly normal. Some of these are shown in the photomicrograph in the plate, while their general distribution is indicated by the stippled circles in the text figure. Now, in this specimen, as in the others, the various parts of the graft lie embedded in a general loose reticulum of mesenchyme, and the sharp distinction between the dermis beneath the epithelium of the skin vesicle and this loose mesenchyme is very clear, giving strongly the impression that the dermis must have come in with grafted ectoderm, and that it has not been formed by the simple aggregation and condensation of the general mesenchymal elements, which certainly originate, in the main at least, from the connective tissue of the chorio-allantois.

Such an impression has little evidential value, and accordingly I made a careful examination of the entire series of sections, with a view to finding dermis developed beneath other epithelia, particularly epithelia of chorio-allantois origin. It should here be stated that all the main components of the graft are adequately represented in the specimen by differentiated structures, the limb anlagen by the cartilages, the ectoderm by the epithelium of the skin and feathers, the splanchnopleur by the gut with its muscular wall. Hence there can be no doubt that the epithelium which covers the graft nodule is of chorio-allantoic origin, and since it projects entirely on the inner face of that membrane, must consist of allantoic endoderm. The chorionic ectoderm is present, lining the opposite side of the membrane from that on which the nodule projects.

No dermis was found beneath the chorionic ectoderm, nor beneath the greater part of the allantoic endodern. There is, however, a definite dermis beneath the endoderm in some regions, especially in the area where the skin vesicle lies very close to it. This dermis varies considerably in its degree of definition and clearness from place to place, being in some regions well organized, like that beneath the skin vesicle, in others merely a thin and vague condensation of the general mesenchyme. An interesting and suggesive point is that wherever a dermis appears beneath the allantoic endoderm, it is connected with either the dermis of the skin vesicle or with the connective tissue sheath (perichondrium) of the cartilages. These connections are made by curious strands and sheets of connective tissue of a type resembling that forming the dermis,

which run from one place to another through the loose general reticulum. The significance of these connecting strands will be considered in the discussion.

The Skin of No. 5. Text figure 7 (sk. ves.).

The skin in this case forms about half the wall of the vesicle (sk. ves). in the text figure. One side of this vesicle is lined by an epithelium of two or three layers of flattened cells, not at all suggestive of skin, and with no dermis underlying it, while the other side has a wall whose epithelium resembles that described in the skin vesicle of No. 4, that is, typical for skin, and beneath which lies a dermis. This dermis differs from that found beneath the skin vesicle of No. 4 in being less fibrous, more densely cellular, and somewhat less abruptly demarcated from the general mesenchymal reticulum. Only two or three young feathers are present.

As in No. 4, the graft nodule projects from the endodermal side of the chorio-allantois, and hence is covered with allantoic endoderm, the chorionic ectoderm covering the membrane on the opposite side. No dermis was found underlying any epithelia other than that of the skin, except for an area under the chorionic ectoderm. There is no strand of connective tissue joining this dermis with that underlying any of the mesodermal parts of the graft, as was the case in No. 4, and there can, I think, be little doubt that this dermis is of chorio-allantoic origin. (See discussion.)

The vesicle in this case is not quite completely closed. It is continuous by means of a narrow channel, with another somewhat irregular cavity, both the channel and the latter cavity having for the most part thin, pavement-like walls. Over part of the latter cavity the wall consists of low cubical cells, perhaps covered with a very thin layer of very flat cells. It seems likely that, during the incorporation of the graft into the membrane, the ectodermal component of the latter became attached by its edges to certain parts of the chorio-allantois, the whole complex then forming the two vesicles and the channel described, with the graft ectoderm forming part of the wall of one vesicle. The origin of the skin dermis will be dealt with in the discussion.

The Skin of No. 2. Text figure 3.

This specimen was dissected to a considerable extent before sectioning, with a view to revealing more clearly the arrangement of the cartilages in the entire cleared preparation. Hence the sections do not show the complete nodule, but only the parts left after dissection.

In parts of the specimen, however, such as covering the distal phalanx of the "digit," and elsewhere, definite skin occurs, with a well-formed dermis and feathers. The specimen has been dissected too much for it to be possible to say whether dermis occurs beneath any of the chorio-allantoic epithelia or not.

DISCUSSION.

The Limb Anlagen.

The grafts contained no vertebral plate tissue, and were removed from the donor before somites had formed in the region opposite the area in which the posterior limbs develop. It is therefore definitely established (saving the possible significance of the abnormal condition of the skeletons in the grafts) that from this stage onwards the presence of vertebral plate tissue is not required for the development of the skeleton of the limbs.

The grafts are undoubtedly very abnormal, and cannot be said to be limbs. They are, however, undoubtedly attempts at the formation of limb skeletons. The question arises whether the abnormalities, so much greater than those which are usually seen in grafts of limb buds taken from older donors, are due to the conditions of grafting or to the absence of any other tissue. We have to decide whether the growth pattern necessary for the development of a normal limb is already determined within the region of the limb anlagen at two days, or whether an essential part of it acts upon the anlagen from outside, from the "embryo as a whole," or from some part of the embryo not included in the graft. In other words, are the abnormalities specifically due to absence of some directing agent in the chick, or are they due to the abnormal conditions of grafting? The matter may be considered in steps: (1) The grafts show that, saving only the possible influence of other tissues included in the graft, such as the endoderm, the cells of the limb anlage are determined at the time of grafting for the formation of jointed and branched cartilaginous structures. (2) Grafts of the limb *bud* of the three or four days chick form limbs, or in the case of fragments of bud, fragments of limbs, which are much closer to the normal than the structures formed by the grafted two days anlage. But the bud, even the young bud, is a far more coherent structure than the two days anlage, consisting of a mass of more or less dense mesenchyme covered with ectoderm, and hence much more likely to be able to retain a normal coherence of its parts than the two days anlage, which is a little piece of flat membrane, very easily torn, and having but little coherence, and hence much less likely to retain the normal relations of its parts when invaded by mesenchyme and blood vessels from the chorio-allantois. Examination of sections of the posterior limb-forming region of a three or four days embryo and of a two days embryo at the stage used will readily convince anyone of this. (3) Some grafts obtained, using four-day limb buds, were even more abnormal than those described in the present paper; it is therefore clear that the abnormal conditions of life as a graft, as distinct from the absence of normal relations with other parts of an entire embryo, are capable of causing very great deviations from the normal even with fairly advanced buds, and the same must therefore, *a fortiori*, be true for the two days anlagen.

There is thus every reason for believing it likely that the abnormalities in

these grafts are due to the abnormal mechanical and other conditions of life as a graft. Nevertheless, we have no conclusive disproof of the possibility that some of the abnormality may be due to the absence of directing influences from other parts of the body.

With regard to the possible influence of the other parts of the graft, it has already been stated that Harrison (1918) has shown for Amphibia that it is the mesoderm which is the active component, the ectoderm being passive and not specific. Even if this be not true for the chick, that is, if the local ectoderm is specifically essential for the further development of limb mesoderm, it would in no way affect our conclusions, since the ectoderm covering a limb is as much a part of the limb as its skeleton, and we are at present considering the entire limb, and not its component parts. The possible significance of splanchnic mesoderm and endoderm must be left open, though, as we have seen in the introduction, there are serious reasons for doubting the influence of the latter at least.

Recent work on the Amphibia has shown that the nervous system is of importance in the development at least of regenerating limbs. Now, in my previous paper (1926) it was shown that the limb bud from three days onward can develop in the absence of any nervous supply, for the chorio-allantois has no nerves, but there was some possibility that the grafts from the two-day chick might have contained a sympathetic apparatus, which might possibly exert some important influence during the third day. This was due to the fact that the primary sympathetic chain, although it is not present as such till the fourth day, may possibly arise wholly or partly from the neural crest, and since the two-day grafts described in that paper included the vertebral plate, they might also have included neural crest material, from which later sympathetic ganglia and dorsal ganglia might have originated. This possibility is excluded in the present series of grafts, because the cut, being lateral to the vertebral plate, could not possibly have included neural crest material. Thus the combined evidence given in this paper and its predecessor demonstrate as conclusively as can be wished that the nervous system plays no essential part in the development of the limbs of the chick. It nevertheless remains possible that in the presence of a nerve supply the grafts might have been less abnormal.

It will have been noticed that the cartilaginous parts of the grafts are far too abnormal in form for it to be possible to say, if one did not know the origin of the grafts, whether the structures constituted attempts to form anterior or posterior limbs. This may be due to the anlage at the time of grafting having been determined only for the formation of limbs, not specifically for the formation of wing or leg. It is, however, at least equally possible that the anlage was at grafting fully determined, but, in the abnormal conditions of life as a graft, was not able to express its potentialities sufficiently fully for the complete differentiation between wing and leg to arise. We remain completely ignorant of the factors bringing about this difference.

It is noteworthy that none of the grafts contain any skeletal muscle, although this was present in the similar grafts described in the previous paper. This is interesting in view of the fact that the latter grafts contained vertebral plate tissue, while the present series do not, suggesting that perhaps the cells forming the muscles of the appendicular skeleton may originate from the somites, in spite of experimental evidence to the contrary from the Amphibia (Byrnes, 1898; Lewis, 1910), and morphological evidence to the contrary for the chick (Patterson).

The Endoderm and Splanchnopleur.

The two outstanding features in connection with the behaviour of these components of the grafts are first the extensive histological differentiation they have undergone, and secondly the fact that two out of the three specimens have formed small closed tubes.

As we have seen, the histological differentiation has involved the development of intestinal epithelia, of circular and longitudinal muscles, and of a subepithelial corium, all in the normal relationships.

It is evidently fair to conclude that small sections of the intestinal anlage obtained from one side, and lacking any medial tissues, and derived from the region of the body of the two days chick behind the last differentiated somite, isolated from anterior, posterior, and medial parts of the anlage, and from all other organs of the chick, except the limb anlage and some somatopleur surrounding it, and some ectoderm with its subjacent dermis, are able to undergo differentiation so as to form the normal tissues of that part of the alimentary tract. In other words, the various regions of the alimentary tract can develop in a high degree independently of other parts of that tract, and of other organs. This, coupled with the complete absence of any indication of an attempt to form a complete alimentary tract, even a complete intestine, makes it evident that we have here another instance of the power of self-differentiation which is well known to be a marked characteristic of many parts of the chick embryo. The alimentary anlage of the chick is thus at two days already a mosaic.

On the other hand, the formation of short, closed tubes, which are obviously little sections of intestine, in two out of the three cases, shows that the fragment has in some degree the power of regulation. It must be remembered that the endoderm and splanchnopleur, when grafted, were small, flat sheets. Had the tissues used remained in a normal chick they would have formed part of the wall of the intestine, and not a complete tubular section. What forces have been responsible for the rolling-up of the flat sheet into a tube?

The general idea which one gleans from text-books, etc., of the mode of closure of the alimentary tract, is that the two opposed sheets of endoderm-splanchnopleur are pushed towards one another by the lateral folds of the somatopleur. That this is very questionable is shown by the fact that sections

may be found, in the four-day chick for example, in which, although the alimentary tract appears as a closed tube, the intra- and extra-embryonic coelomes are in free communication. In other words, the closure of the splanchnopleur precedes that of the somatopleur, and so can hardly be caused by the latter. There seems to be no information available upon this question, and it is evident that from the present experiments one can only make suggestions. It is clear in the first place that the rounding up in the two cases might be due simply to mechanical factors in the chorio-allantois. There is no reliable evidence either for or against this possibility. But if this is not so, then it is clear also that the forces involved in the folding off of the embryo from the yolk-sac, and similar factors, can have played no part in the development of the grafts. The remaining possibility, which seems to me the more probable, is that the assumption of the tubular form is due to differential growth rates in the endoderm and the splanchnopleur. If the latter grows more rapidly than the former it is evident that a tube must be formed, with the endoderm lining the lumen. I hope to test this hypothesis by means of more grafts and by cultivation of small fragments of intestinal anlagen *in vitro*.

Graft No. 6, in which the intestine is turned inside-out, at first sight seems to speak quite against this suggestion. In reality I think it is favourable rather than otherwise. It differs from the other two specimens, in that it is attached to the chorio-allantois only at the base, the greater part being suspended free in an internal cavity of the membrane. It is quite clear that the anlage became attached to the chorio-allantois by the mesodermal side, the endodermal side facing into a cavity in which this part of the graft became enclosed. Then differentiation and growth continued, and the mesodermal component, growing rapidly, tried as usual to force the endodermal part into a tubular form. This was impossible, because both endoderm and splanchnopleur were attached to the chorio-allantois over the entire mesodermal side and at the edges in such a manner that the surrounding regions of the chorio-allantois would have had to be pulled over into a tubular form along with the closing intestine, and this the growth energy of the splanchnopleur was unable to accomplish. Nevertheless, the differential growth rates continued, and the splanchnopleural parts were forced to expand somewhere, and this was accomplished by pushing the endoderm out into the internal space as a finger-shaped process. This view finds support in the flat, unfolded epithelium of this specimen, so strongly contrasted with the much-folded epithelia of the other two. On this view, then, the final form taken by a grafted fragment of intestine is determined by (a) the different growth rates of the endodermal and mesodermal components, and (b) by the conditions of grafting in each individual case.

A direct extension of this hypothesis to cover the normal closure of the gut would at this stage be perilous in the extreme, especially since the gut closes before the muscular layers differentiate as such. It is nevertheless perhaps

significant that the gut is surrounded at least very soon after closure by a dense layer of mesenchyme, in which the muscles evidently arise.

I hope to consider the whole question more fully in a subsequent communication, with more evidence.

The Ectoderm and Dermis.

The main feature of interest in this connection is the presence of the well-developed dermis. It is well known from morphological studies of the history of the somites (Engert, etc.) that the dermis is derived, at least in part, from the dermatome. The latter is seen first as a definite epithelial lamella on the outer face of the somite. This epithelium then breaks up into its component cells, which thus take on a mesenchymal character, and wandering away dorsally and ventrally, give origin to the dermis of at least the dorsal and dorso-lateral regions. It is not possible by ordinary morphological methods to discover whether or not the dermis underlying the ectoderm covering the lateral plates and limbs is derived from the dermatome or from cells derived from the superficial layers of the somatopleur, as the two groups of cells mix and become indistinguishable. It is, nevertheless, frequently assumed that the former is the case. The dermis of the skin found in these grafts must have had one of two origins: either it is derived from the graft mesoderm or from chorio-allantoic mesoderm. The chorio-allantoic mesoderm is made up partly of the somatopleuric mesoderm of the chorion and partly of the splanchnopleuric mesoderm of the allantois; that originating from the graft must be derived either from somatopleur or splanchnopleur of the small lateral region grafted, for, since the grafts were made at a time when somites had not appeared opposite the region grafted, and since they contained no vertebral plate tissues, no other mesodermal elements can have been present. It is therefore impossible that the dermis found in the grafts can have been derived from dermatome material.

The main point is then established that the dermis found in the grafts is not derived from the dermatome. It therefore becomes of importance to know whence it is derived. The following considerations suggest that it comes from the graft somatopleur: (1) There is no evidence to the contrary. (2) It is present, in a well-developed state, under all three ectodermal vesicles. (3) Dermis is not usually found under either ectodermal or endodermal epithelia of the chorio-allantois. (4) In the case (No. 4) in which an extensive development of dermis has occurred under the endoderm of the chorio-allantois, this dermis is connected by a number of dense strands of connective tissue cells with either the dermis of the skin vesicle, or with the perichondrium of the cartilage, or with both. (5) In No. 5 there is a well-developed dermis beneath the skin vesicle, but none under any of the chorio-allantoic membranes, except a small area under chorionic ectoderm. (6) Rendering the argument almost conclusive is the presence in all three skin areas of definite young feathers. The development of feathers has

never been observed in the chorio-allantois except after the grafting of embryonic tissues normally destined to form feathers. Both Hoadley (1926) and I have observed the latter. Hoadley further observed that epidermis alone, without dermis, does not form feathers, but will do so if dermis is present, and there is every reason for believing, with Hoadley, that this dermis is of graft origin. Thus it is reasonable to conclude that chorio-allantoic mesoderm is unable to form feathers, even though epidermis is present. Hence the dermis in my specimens must be of graft origin, in other words, derived from intra-embryonic somatopleur.

It cannot be said that chorio-allantoic mesenchyme is incapable of forming dermis. At least one area of chorionic ectoderm has a dermis beneath it in No. 5, and this dermis is almost certainly derived from the chorio-allantois. It nevertheless remains a fact that a dermis is not usually formed, and that chorio-allantoic mesoderm cannot form feathers even in the presence of epidermis.

The weight of the evidence thus favours the view that the dermis of the graft skin is derived from cells originating in the graft. These must have been derived from the somatopleur of the graft.

We may then conclude that the dermis has two origins. That underlying the epidermis of the dorsal and dorso-lateral regions of the body is derived from the dermatomes of the somites. This appears to be adequately established by the morphological evidence. That, on the other hand, which underlies the epidermis of the limbs, and probably of the flanks and ventral surface, is derived from the somatopleur.

SUMMARY AND CONCLUSIONS.

1. Chorio-allantoic grafts were made of lateral fragments of the two days chick, taken from the region forming the posterior limb, and removed by an anterior cut posterior to the most posterior somite formed, by a posterior cut a little anterior to the level of the anterior end of the relic of the primitive streak, by a median cut lying lateral to the vertebral plate, and by a lateral cut at or near the lateral edge of the pellucida.

2. The grafts show cartilaginous structures evidently to be interpreted as attempts at the formation of limbs. It is thus demonstrated that no essential influence is exerted upon the development of the limbs by the somites, or, from the stage used onwards, by the unsegmented vertebral plate.

3. Reasons are adduced in favour of the view that if the limb anlage at two days could be completely isolated from other parts it would be found that self-differentiation would occur, in other words, the anlage of the posterior limb is already determined at this stage.

4. The nervous system exerts no essential influence on the development of the limbs of the chick.

5. The endodermal and splanchnopleural components of the grafts have in three cases given rise to short sections of intestine, with epithelium, corium, circular, and longitudinal muscle layers. Small fragments of the region of the alimentary tract are thus able to develop in the absence of all other regions of the tract. There is no indication of any attempt at regulation towards the formation of any region of the intestine other than that represented in the anlage grafted. On the other hand, some degree of regulation is shown by the rounding up of two out of the three specimens to form short tubes. Reasons are advanced favouring the view that this rounding, and perhaps also the closure of the gut in normal development, is due to differential growth rates in mesodermal and endodermal components of the gut, the former growing more rapidly than the latter, and so causing the whole structure to become a tube. The third specimen, in which the intestinal epithelium has developed as the outer covering of a finger-shaped process, which contains internally corium, circular muscle, and an axis of longitudinal muscle, is shown to be explicable by the same hypothesis.

6. In several of the grafts there is developed definite skin with a well-organized dermis and young feathers. This dermis cannot have originated from the dermatome of somites, and it is shown that it almost certainly arose from superficial cells of the grafted somatopleur. It is pointed out that the theory according to which the dermis of the skin originates entirely from the dermatomes is based upon insufficient evidence, and that the experimental evidence here presented speaks against this view. It is concluded that the dermis of the dorsal and dorso-lateral regions arises from the dermatome, the remainder of the dermis from cells of the local somatopleur.

In conclusion, I wish to express my gratitude to the late Professor Launcelot Harrison, of this department. Through the several years during which I have worked here he was always ready and willing to give whatever help was possible to anyone engaged in any kind of research. I have to thank especially my wife, who assisted in carrying out the experiments, Professors Burkitt and Stump (of the Department of Anatomy of this University) for much assistance, and particularly for the services of their technical staff, and Miss Molly Garde, also of the Anatomy Department, for taking the photomicrographs shown in the plates.

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STUDIES ON THE GOLGI BODIES OF A COELENTERATE

by

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INTRODUCTION.

The studies of various investigators have resulted in demonstrating the wide distribution of the internal apparatus of Golgi among the cells of the higher metozoa. Less, however, is known concerning their morphology and behaviour in the lower organisms, and in order to help elucidate their nature, this investigation is directed towards determining the occurrence and peculiarities of these cytoplasmic inclusions of a common coelenterate.

MATERIAL AND TECHNIQUE.

The material used in this research was *Hydra viridis*, which was obtained from the laboratory aquaria. For rendering visible the Golgi elements the following technique was employed during investigation:

All material was fixed for a period of 8 hours in Mann's osmo-sublimate solution, which was made of a freshly prepared mixture of equal parts of a 1% osmic acid and a saturated solution of corrosive sublimate in normal saline. The organisms were next transferred to a 2% osmic solution for a period of 14 days. The material was afterwards washed out in running tap water for 24 hours, brought up through the alcohols, cleared, and finally embedded in paraffin. The sections were cut 3–5μ in thickness, and stained by the Heidenhain iron haematoxylin process, which rendered the Golgi elements deep purple and nearly black in colour. In order to test the lipoidal nature of the Golgi apparatus, several organisms were fixed for several hours in common lipoid solvents, such as acetic acid, alcohol, and ether; and this material was then embedded in paraffin, sectioned stained, and then temporarily mounted in glycerine. After such treatment the Golgi bodies were quite incapable of absorbing the haematoxylin stain.

Examination with high magnifications of stained sections, cut transversely

through the gastro-vascular cavity of the coelenterate, reveals various intracellular structures present in the cytoplasm of the ectodermal cells of the organism, which stain a deep purple after treatment with the Heidenhain process.

After a more detailed and topographical study of these cytoplasmic inclusions, during the resting stage of the cell, two distinct and separate cell structures can be detected as follows:

(a) A dense network lying in the protoplasm in close connection with the outer surface of the nuclear membrane.

(b) A large crescent-shaped body, encircling the nucleus, and also lying in close connection with it.

For the following reasons these dimorphic intracellular structures are identified as the Golgi apparatus:

(1) Their morphology is identical with the Golgi elements of the higher organisms.

(2) Their reaction to certain lipoid-solvents and stains is similar to that of the Golgi apparatus of other animal cells.

OBSERVATIONS AND DISCUSSION.

When the ectodermal cells of the coelenterate are examined with high magnification, during the resting stage, the two distinct kinds of Golgi elements may be seen quite clearly lying in the cell cytoplasm in close connection with each nucleus. (See figures 1, 2.)

A more detailed examination of the former type of intracellular structure shows that the network is composed of a more or less dense meshwork of numerous anastomosing threads, which resemble very much the Golgi network usually described in the somatic cells of the vertebrata. The appearance of this network-like structure in the ectodermal cells of *Hydra viridis* is of interest, as

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DESCRIPTION OF PLATE.

All figures are drawn from material fixed in Mann's osmo-sublimate solution, and stained with Heidenhain's iron haematoxylin.

Fig. 1. Section through ectodermal cell of Hydra viridis, showing the crescent shaped Golgi body lying in close connection with the nucleus. The nucleolus is clearly visible.

Fig. 2. Section of same, showing the Golgi network in region of nucleus, and in close connection with outer surface of nuclear membrane.

Fig 3. Longitudinal section through ectodermal cell of Hydra undergoing division. The cell is in the telophase. Note how the Golgi bodies have distributed themselves more or less evenly at opposite poles of dividing cell.

Fig. 4. Cell in prophase. Crescent shaped body has migrated from region of nucleus, and taken up position midway between nucleus and periphery of cell.

Fig. 5. Same. The Golgi network which has migrated away from region of nucleus, and is observed undergoing fragmentation.

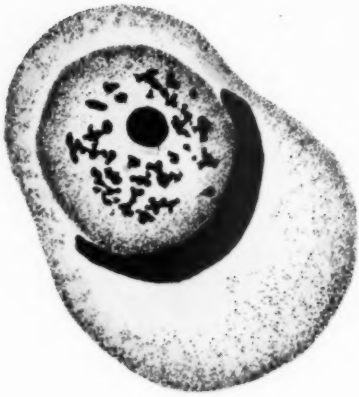

Figure 1

Figure 2

Figure 3

Figure 4

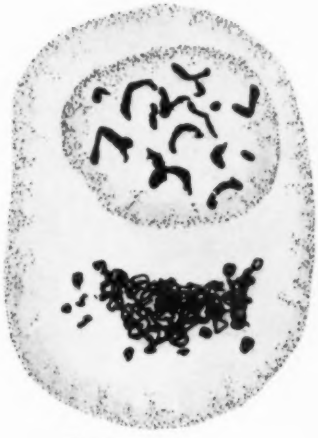

Figure 5

the Golgi apparatus in the lower metozoa is generally represented in the form of isolated rods or disconnected fragments.

Observations on the latter type of Golgi bodies show that these structures take the form of a crescent, and exhibit a compact appearance. (See figure 1.)

On the Relation of the Golgi Apparatus to the Nucleus.

Topographical studies upon these two types of Golgi bodies during the resting stage of the ectodermal cells, always reveals them in the region of the nucleus and lying in close connection with the outer surface of the nuclear membrane. (See figures 1, 2.)

In a previous paper (4) I have pointed out that similar aggregations of mitochondria around the nuclear membrane, as well as around the periphery, such as are seen in many cells, may be a surface tension phenomenon which is apparently dependent upon the phosphatidal nature of these bodies.

According to some writers (3) the chemical composition of the Golgi apparatus is said to be very much like that of mitochondria, i.e., proteid in some way linked with lipin material, and mitochondria like the Golgi bodies are also readily attacked by acetic acid, alcohol, ether, and other lipoid-solvents.

It is therefore possible that the aggregation of the Golgi elements at the nuclear-cytoplasmic interface may be related to their lipoid components, and such a conclusion would doubtless help to explain the frequent occurrence of these bodies within the region of the nucleus.

When cell division is about to occur the Golgi elements are observed to migrate from the region of the nucleus towards the periphery of the cell and take up a position midway between the nucleus and the outer boundary of the cell.

During the early prophase, when determining the behaviour of the apparatus during mitosis in various cells, several authors have detected this phenomenon.

It is of interest to note that Ludford and Gatenby (6), when observing the behaviour of the Golgi bodies during cytokinesis in germ cells, conclude that the lipoids of the Golgi substance are withdrawn at the prophase. Assuming the aggregations of the Golgi elements around the nuclear membrane of the resting cell to be a surface tension effect, this withdrawal of the lipin material at the commencement of cytokinesis may possibly explain the migration of these bodies from the region of the nucleus towards the periphery of the cell. Recently King and Gatenby (5), while studying the Golgi bodies of a Coccidian, noted that the apparatus always lies away from the nucleolus. I have not been able to confirm this phenomenon during this investigation.

Behaviour of the Golgi Elements during Cytokinesis.

(a) *Crescent-shaped Body.*

Shortly after the crescent-shaped body migrates from the region of the nucleus during the early prophase, it commences to undergo a process of fragmentation by breaking up into several small masses, which later separate, forming irregular-shaped bodies within the protoplasm of the cell. (See figure 4.) This phenomenon is of interest, as some writers are sceptical as to whether the Golgi apparatus is capable of multiplying by growth and division, while others hold that these bodies arise *de novo*. When the early telophase of the ectoderm is examined, the Golgi fragments have sorted themselves into more or less equal halves and taken up a position at the opposite poles of the dividing cell. (See figure 3.) Observations upon newly-formed daughter cells show that these bodies aggregate in the cytoplasm in the nuclear region of the cell, where they apparently fuse and transform into the original body.

(b) *Network Body.*

In the case of the Golgi network, this structure during the late prophase is observed to break up into a number of small, irregular-shaped bodies, which spread out through the general protoplasm of the cell. (See figure 5.) Similarly, these bodies distribute themselves more or less equally at each pole of the dividing cell, and at the close of cell division these separate elements aggregate to give rise to the original structures seen in the resting cell.

Function.

Very little is known concerning the behaviour and physiology of the Golgi elements to justify any lengthy discussion at the present moment. Recently several authors have gone so far as to suggest that these cell inclusions are artefacts, their conclusions being based upon the fact that these structures cannot be detected within the living cell *in vitro*, nor can they successfully be stained *intra vitam;* as the Golgi apparatus exhibits a definite behaviour during mitosis these conclusions seem hardly justified.

Nevertheless, it is of interest to note the recent work of Ludford (7), Bowen (2), and Nassonov (8), who contend that the Golgi apparatus plays a very important part in secretory processes.

The two latter authors have brought forward evidence which suggests that the secretory granules in the glands of salamanders are the products of the Golgi apparatus, and that each granule arises in close connection with the Golgi reticulum. Later these secretory granules migrate into the protoplasm of the cell, where they increase in size, and are finally passed into the lumen of the glands.

Nassonov (9), while studying the behaviour of the Golgi apparatus in the epididymis, describes how certain granules, which he interprets as modified

mitochondria, make their appearance in relationship with the network of the apparatus. While observing the mitochondria of the ectodermal cells of *Hydra viridis* I was unable to detect any topographical relationship between the Golgi apparatus and these granules.

SUMMARY.

1. Dimorphic Golgi bodies have been detected within the ectodermal cells of *Hydra viridis*.

2. Aggregations of these bodies around the outer surface of the nuclear membrane is a surface tension phenomenon, apparently dependent upon their lipoidal nature.

3. The behaviour of the Golgi apparatus during cytokinesis is also described.

During the early prophase these dimorphic elements migrate from the region of the nucleus towards the periphery of the cell, where they undergo fragmentation, resulting in the formation of numerous small, separate bodies, which are later distributed among the daughter cells.

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THE BASAL METABOLISM OF SOME AUSTRALIAN ABORIGINES

by

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The importance of racial factors in determining the level of basal metabolism has not yet been correctly assessed. In most investigations which bear on the subject the complicating effects of climate, diet, activity, and general conditions of life have not been excluded. It is rather difficult to disentangle the various influences at work, because, apart from climatic influences, diet and general conditions of life are themselves, to some extent, racial characteristics. A comparison of members of two races under identical conditions may mean that one of the races is not being examined in its accustomed environment. This circumstance may have an influence on the value obtained for the basal metabolism. In most cases in which comparisons have been made between Europeans and persons of other races, the subjects of examination were living their accustomed lives with regard to diet and activity.

The classical work on this subject is that of Eijkman (1896), in which he compared the basal metabolism of eleven Dutchman living in Batavia with that of twelve native Batavians. Eijkman found no significant difference between the average basal metabolism of his European and that of his native subjects. This work was carried out when methods for the determination of basal metabolism were still in their infancy, and Ozorio de Almeida (1924) has shown that the results are high, owing to a systematic error. This fact is equally applicable to the results obtained on both series of subjects, and Eijkman has performed a sufficient number of determinations to exclude any material effect of chance errors on his average figures. His conclusion as to the similarity of the average basal metabolism of the members of the two races therefore does not seem to have been invalidated. The two series of subjects were leading their accustomed lives. In addition the possible effect of a response to a changed environment is not excluded, as none of the European subjects had been born in the tropics.

Ozorio de Almeida (1919, 1, 2; 1924, 3) determined the basal metabolism of eighteen native-born Brazilians of European stock and of ten of negro stock.

He found the basal metabolism of his negroes to be on the average 3% higher than that of his Europeans but 17% lower than the American (Aub–Du Bois) standards. The subjects were living under their accustomed, dissimilar conditions, but any effect due to lack of acclimatization was avoided.

Earle (1922, cited by Macleod *et al.*, *loc. cit.*, 1928, personal communication) observed that the basal metabolism of Chinese in Hongkong was distinctly lower than that of residents of European birth. Here again, although the climatic conditions were similar, the possible effects of different habits of life and of imperfect acclimatization were not excluded.

Macleod, Crofts, and Benedict (1925) appear to be the only investigators who so far have examined the basal metabolism of members of different races under strictly identical conditions of climate, diet, and activity. They found that the average value of the basal metabolism of seven Chinese and two Japanese women in two American colleges was 5% lower than the average value obtained for young American women in the same institutions, or 10% lower than the Aub–Du Bois standards. Even in this investigation the effects of incomplete adaptation to their new environment may have been a complicating factor, as the Oriental subjects were not American born, and had been away from their homes for periods ranging from fifteen to fifty-two months.

Takahira (1925, cited by Du Bois, *loc. cit.*) appears to have obtained average figures for the basal metabolism of sixty-nine Japanese men and forty-three Japanese women, which were respectively 5·5% and 7·3% below the American standards.

Okada, Sakurai, and Kameda (1926) observed that the average basal metabolism of forty-two young Japanese men was only 2% lower than the American standards, while the average figure for eleven young women was only 0·7% below the same standards. The subjects were students and nurses. In these comparisons the climatic factor, as well as the factors due to different habits of life, have, of course, not been excluded, but the findings of these Japanese observers do not disagree materially with those of Macleod, Crofts, and Benedict *(loc. cit.)*.

Turner (1926) measured the basal metabolism of students of a number of different races in Syria. The most homogeneous groups were that of the Armenians and that of the Egyptians. The former had an average value of the basal metabolism less than 1% below that of the American residents. The latter group had an average basal metabolism 6% below that of the Americans. This group showed the lowest average value observed. The average value for the Americans themselves was within 0·5% of the Harris Benedict standards.

PRESENT INVESTIGATION.

In the present investigation of the basal metabolism of members of a non-European race, measurements have been made on Australian aborigines. As

far as could be ascertained by observation, and inquiry into their family histories, the subjects were full-bloods. With regard to food, clothing, and housing, the subjects were living under civilized conditions. The principal difference between their mode of life and that of the white inhabitants is the higher degree of activity to which the latter are accustomed.

This report is restricted to the results obtained upon eight adult males, whose confidence was very satisfactorily established. These subjects relaxed completely during the experimental periods; indeed, in several instances care had to be taken to keep them awake during the experiments. The ages of the subjects ranged from 35 to 65 years, but several men did not know their ages accurately.

The subjects were not examined medically before a determination was made. None showed any obvious physical abnormality. All were apparently in their usual state of health. In particular, no evidence of goitre, such as could be seen in several of the women, was observed in any of the male subjects. The mouth temperatures of the subjects were observed before examination, to make sure that no measurements were made upon persons showing temperatures above normal, i.e., over 37·20°C.

The various subjects showed considerable difference of physique. One of them (No. 1) was a very powerful man of quite exceptionally great muscular development. He had been a professional boxer. Of the others, two were of good physique (Nos. 2 and 4), while the rest were more or less undersized as compared with American standards, as shown by the figures for their heights and weights.

The examinations were made at the Aborigines' Reserve at Runnymede (latitude 28° 42′ S., longitude 153° 0′ E., height above sea level 154 feet) during the month of February. The dry-bulb temperatures ranged from 23·6°C. to 27·8°C. (average 25·3°C.); the relative humidity ranged from 69% to 94% (average 80%) during the period of examination. The climate during this period was therefore warm and humid.

The men living at the Aborigines' Reserve are required to do a certain amount of work to entitle them to receive a weekly ration. The requirement, however, is by no means exacting, and is satisfied by the performance of an occasional task during the week. If their average physical activity be described as that associated with light work it will not be under-estimated. A great deal of their time is spent lounging about or sleeping. In this respect subject No. 1 was again exceptional, and displayed considerably more energy and initiative than the rest.

Each man at the reserve receives the following weekly ration when entitled to it: Flour, 8 lb.; sugar, 2 lb.; fresh meat, including bone, 2 lb.; salt meat, 3 lb.;

tea, ¼ lb.; baking power, ¼ lb.; and a daily allowance of fresh milk. The calorific value of the flour and sugar alone gives a daily average of 2,360 calories. The calorific value of the meat is more difficult to judge, but it is not likely to be less than enough to bring the daily average above 3,000 calories. This ration is supplemented by food bought with the earnings from the subjects' occasional employment.

The activity of these men was not greater than that of a number of male students whose diet Wardlaw (1922) found to contain a daily average of 2,170 calories. The present subjects thus had at their disposal a diet abundant for their needs. In most instances, however, they used their food very wastefully, a period of plenty immediately after the issue of the ration being followed by a period of want during the rest of the week.

METHODS.

The present observations were begun early in the morning, as soon as possible after the subject had got out of bed. The huts in which the aborigines slept were provided with wooden shutters. These were closed at night, as well as the doors. The huts therefore did not become light early in the morning, and the subjects were not early risers. In many instances they had to be roused from sleep for the examination. The determinations of basal metabolism were made after a period of from 14 to 16 hours from the last meal.

The determinations were made in a tent within 100 yards of the huts, and downhill from them. The subjects were thus able to reach the place of examination with a minimum of exertion. They were made to lie quietly on a stretcher for half an hour before any observations of basal metabolism were begun. During this period their mouth-temperatures were taken, and they were allowed to familiarize themselves with the collecting portion of the apparatus, if they had not already done so. It was found that by encouraging the subjects to examine and handle the apparatus before the observations any feeling of apprehension which they might have experienced was largely avoided, and they were obtained in a state of psychical as well as physical rest.

After the preliminary period one or more blank experiments were carried out, until the pulse and respiration of the subject became steady. The variations of pulse-rate during the periods of examination did not exceed five beats per minute in either direction. The corresponding variations of rate of respiration did not exceed four movements per minute. The specimens of expired air were collected for periods of 10 minutes.

What might seem excessive precautions were taken to get the subjects in a state of complete physical and psychical rest. It seems to the present investigators, however, that it is hardly possible to expend too much care in reaching

this condition. The success with which this condition was reached with these subjects was shown by their readiness, already referred to, to fall asleep during the observations. The experiments of Wishart (1927) indicate that even with Europeans in a cold climate figures for the basal metabolism, which are considerably (20%) lower than the usual standards, are obtained when special precautions are taken. Indeed, the whole question of the definition of the basal state, as discussed by Krogh and Lindhard (1920) and others, seems to be raised again by their experiments.

The collection of expired air for analysis was made through the usual type of rubber mouthpiece, fitting between the lips and teeth, and connected with rubber diaphragm valves. A length of about 40 cm. of 2·5 cm. rubber tubing was attached to the inspiratory side of the valve, so that any leakage through it would be rebreathed. It is not so easy to exclude the possibility of leakage round the mouthpiece, but care was taken to see that this was held firmly between the lips and teeth. The nose was clipped by means of short lengths of lead-covered double electrical conduit wire, the ends of which were protected with rubber tubing, and bent so as to squeeze the nostrils together. The specimens of expired air were collected in a Douglas bag; their volumes were measured with a wet gas meter. The samples for analysis were collected over mercury in 100 cc. glass syringes (Martin). The samples were analysed in the portable Haldane apparatus.

RESULTS.

In Table 1 the following measurements and calculated results are recorded: (1) The ages of the subjects in years; (2) their heights without boots to the nearest 0·5 cm.; (3) their weights: the subjects were weighed lightly clad to the nearest 0·5 kg., a deduction of 2 kg. has been made for clothing; (4) the area of body surface derived from Janet's nomogram (1922), based on the formula of Du Bois and Du Bois (1915); (5) the volume of air expired in 10 minutes reduced to N.T.P. and corrected for water vapour; (6) the percentage of carbon dioxide in the expired air; (7) the corrected percentage of oxygen absorbed; (8) the respiratory quotient, not corrected for protein metabolism; (9) the volume of oxygen absorbed per minute at N.T.P.; (10) the number of calories per sq. m. per hour corresponding to the oxygen absorption at the existing R.Q.; (11) the Aub– Du Bois standards for the subjects; (12) the calculated heat evolution per sq. m. per hour expressed as a percentage of the Aub–Du Bois standard; (13) the calculated heat evolution per sq. m. per hour expressed as a percentage of the standards based on the height-weight-age tables of Harris and Benedict (1919).

TABLE 1.

Basal Metabolism of Male Australian Aborigines.

Subject.	Age, years.	Height. cm.	Weight. kg.	Area sq. m.	Volume exp. in 10 min.	CO %	O % abs. corr.	R.Q.	O abs. per min.	Calories per sq. m. per hr. Standard.	Calories per sq. m. per hr. Observed.	B.M. as p.c. standard. A.–D.B.	B.M. as p.c. standard. H.–B.
1	40	179	96	2.15	69.0	3.82	4.31	0.886	297	40.5	38.5	105	104
					69.6	3.86	4.34	0.890	302	41.5		107	106
2	54	175.5	75	1.91	42.2	3.28	4.34	0.756	183	27.3	37.5	73	78
					35.5	3.80	4.73	0.805	168	25.4		68	72
3	65	169	61	1.70	47.3	2.42	2.87	0.844	136	22.5	36.5	62	70
					39.2	2.43	3.11	0.782	122	20.6		56	64
4	45	167.5	67.5	1.75	41.3	3.55	4.00	0.888	165	27.8	38.5	72	74
					36.7	3.85	4.49	0.858	165	27.6		72	74
5	35	173	61.5	1.73	46.1	4.25	5.18	0.821	239	24.5	39.5	62	66
					45.5	4.59	5.35	0.858	243	24.3		62	65
9	40	161	44	1.43	27.3	3.18	4.01	0.793	110	21.5	38.5	56	61
					26.5	3.21	4.20	0.764	111	22.2		58	65
12	50	164	54.5	1.59	20.7	4.11	5.55	0.741	115	20.6	37.5	55	61
					23.9	3.91	5.14	0.761	123	22.0		59	65
16	55	173.5	61.5	1.73	39.9	3.34	4.15	0.805	166	27.6	37.5	74	78
					34.9	3.44	4.25	0.810	153	25.4		68	71
15	16	164.5	45	1.47	32.5	4.02	4.41	0.912	143	47.0	43.0	109	
					30.6	3.86	4.77	0.809	146	48.5		113	

The most striking feature of these results is the very low level of most of the values of the basal metabolism. Seven of the eight results obtained from full-bloods lie between 72% and 54·5% of the Aub-Du Bois standards. The average value is 69·3% of this standard.

Subject No. 1, whose basal metabolism is slightly above the standard, was quite exceptional, both with regard to his muscular development and to the evidence which he gave of energy and initiative.

The figures for the last subject (No. 15), who was a half-caste, have not been included in the average, but are recorded for comparison. They lie well above the figures of the full-bloods of similar physique.

The respiratory quotients of the full-bloods range from 0·741 to 0·888. The average value is 0·817. These figures give no indication of any abnormality in the respiratory exchange.

DISCUSSION.

A number of determinations of the basal metabolism of white Australian students has been carried out by Hindmarsh (1927). Her figures for male subjects range from 115% to 70·6% of the Aub–Du Bois standards, and have an average value of 91·1%. The respiratory quotients of these subjects range from 0·687 to 0.984, and have an average value of 0·850.

Although this average figure for white Australians is materially lower than the American standards, it is considerably higher than the average figure found for the aborigines. The results of Hindmarsh were obtained at Sydney (lat. 33° 52′ S.). The average annual temperature of Sydney is 3°C. lower than that of Casino, a town about 10 miles south of Runnymede, and the place nearest to it for which annual temperatures are available. In making any comparison between these figures for the white Australians with those for the blacks, it must be borne in mind that the difference observed may be partly the result of the difference of climate.

It is interesting to note that the values found by Miss Hindmarsh (unpublished results) for the basal metabolism of five male subjects whose ages ranged from 16 to 31 years, and who were 3/8 to 5/8 crosses between white and black Australian stocks, ranged from 86·3% to 98·2% of the Aub–Du Bois standards. The average value of their basal metabolism was 93·1%, or not significantly different from that of the white subjects. These figures, together with the present results obtained with a half-caste, seem to indicate that admixture of approximately 50% of white blood brings the basal metabolism up to that of the full-blood whites examined locally.

Three factors, aside from specific racial factors, may combine to account in part for the low values observed for the basal metabolism of these Australian aborigines: (1) Their lethargic habit of life; (2) the climate in which they live; (3) their physical development.

(1) An influence of habitual physical activity in basal metabolism has been observed by several investigators. Takahira *(loc. cit.)*, for example, on arranging his subjects according to their physical activity found that there was a variation of about 12% between the average figures for the most active class and that for the least active class.

Similar, but more striking, results were obtained by Ozorio de Almeida (*loc. cit.*, 1). He found that the basal metabolism of his most active subject, who was accustomed to vigorous daily exercise, fell but little short of the Du Bois standard. His least active subject, a man who took practically no physical exercise, had a basal metabolism of only 65% of this standard. Observations made upon another subject are even more remarkable. At the beginning of the investigation this man was taking hardly any exercise, and had a basal metabolism 57·6% of the American standard. He then changed his habits, and entered upon a regime of daily exercise. His basal metabolism rose from month to month, until at the end of a year it was 81% of the American standard. The weight of the subject did not change significantly during the period of the experiment. These results seem to indicate that the effect of physical activity may be more pronounced in a warm than in a temperate climate.

(2) The climatic conditions under which the present results were obtained were similar to those under which Ozorio de Almeida made his observations.

The mean temperature of the hottest month in Rio de Janeiro (lat. 22°54′ S.) is given by him as 26·4°C., and the mean annual relative humidity as 78%. The average temperature observed during the present experiments was 25·3°C., and the average relative humidity was 80%. The mean monthly temperature of Casino for February is 24·0°C. The mean annual temperature of Casino, however, is 19·7°C., or 3·6°C. lower than that of Rio de Janeiro (23·3°C.). The mean annual temperature of New York (10·9°C.) is 8·8°C. lower than that of Casino.

(3) No figures are available for the average weight of Australian aborigines of different heights and ages. It is difficult, therefore, to form a just estimate of the extent to which the present subjects are under or over weight. The different individuals may be compared one with another, however, by reference to some arbitrary standard. For this purpose the figures published by the Association of Life Insurance Directors and Actuarial Society of America (see Hunter) (1912) have been used. A deduction of 4·5 kg. has been made from these figures to allow for the weight of clothing.

It has been shown by several workers, notably by Zuntz and Loewy (1918), and by Benedict, Miles, Roth, and Smith (1919), that a lowering of the body-weight is accompanied by a lowering of the basal metabolism. Ozorio de Almeida (*loc. cit.*, 1) found that the average basal metabolism of his subjects who were lighter than their "theoretical" weights was distinctly lower than the average basal metabolism of his subjects who were heavier than their "theoretical" weights.

In the following table (Table 2) are shown the observed and the "standard" weights of the present subjects, and their observed weights and basal metabolism expressed as percentages of the standards. The results are shown graphically in the accompanying diagram (figure 1).

TABLE 2.

Relative Weight and Relative Basal Metabolism of Male Australian Aborigines.

Subject	Body weight		Obs. wt. × 100 Stand. wt.	Obs. B.M. × 100 Stand. B.M.	
	Observed	Standard		A.–D.B.	H.–B.
1	96	77	125	106	105
2	75	74	101	70·5	75
3	61	71	85	59	67
4	67·5	66	103	72	74
5	61·5	68	91	62	65·5
9	44	61	72	57	63
12	54·5	64·5	85	57	63
16	61·5	72	86	71	74·5
15	45	53·5	86	111	—

Although the results in the above table are few, the tendency for low relative weights to be associated with low relative basal metabolism is unmistakable. This trend of the basal metabolism with the body weight is shown graphically in the accompanying diagram (figure 1). These results seem to indicate that the basal

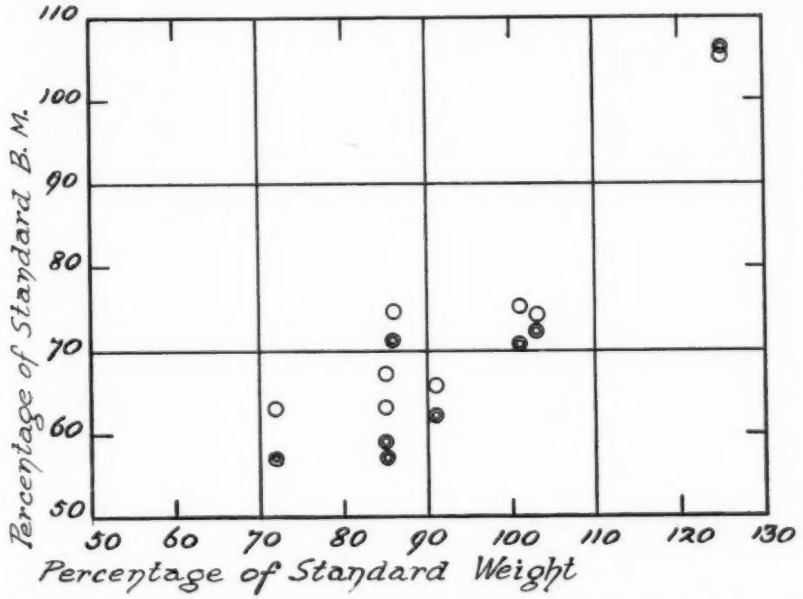

Figure 1. Relation between relative weight and relative basal metabolism of Australian aborigines.

○ According Aub-Du Bois standards.

◉ According to Harris-Benedict standards.

metabolism of an Australian aboriginal, who is not under weight according to the above standards, would be about 80% of the standard values of Aub and Du Bois. The results for the half-caste subject fall outside of the limits of this diagram.

SUMMARY.

The average basal metabolism of eight full-blood male Australian aborigines was found to be 69·3% of the Aub–Du Bois standards.

The basal metabolism of the individuals appears to vary with their relative body-weights.

In conclusion, we desire to express our thanks to Professor H. G. Chapman, who placed the necessary apparatus at our disposal, and gave us essential help

in other ways; to Professor Radcliffe Brown, Associate Professor Priestly, and to the Protector of Aborigines and the officers of his department, who facilitated the work, in particular to Mr. J. P. Howard, whose assistance in the field was invaluable.

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ANAPHYLACTIC EXPERIMENTS WITH EXTRACTS OF LIVER FLUKE *(FASCIOLA HEPATICA)*

by

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Weinberg (1909) and, independently, Paccanaro (1909) first showed that extracts of liver fluke could be used as antigen in specific complement fixation reactions with the sera of infested sheep. These observations were confirmed by Servantie (1921) and by N. H. Fairley and Williams (1922), both of whom used alcoholic extracts of fluke as "antigen."

Guerrini (1912) extracted "nucleo-protein" from fluke, and used it as a control in anaphylactic experiments with "nucleo-proteins" obtained from other sources. Turner (1925) showed that a 1% extract of dried liver fluke in saline could be used to sensitize guinea-pigs, which when tested on the 13th and 32nd days by the isolated uterus method were insensitive to host protein (sheep), but gave sub-maximal anaphylactic reactions with saline extract equivalent to 1.0 and 5.0 mgm. of dried parasite in a volume of 50 c.cm.

The observations recorded here were made in the hope of throwing fresh light on the nature of the substances in helminthic extracts, which act as "anaphylactic antigens" in the sense that they can be used both to sensitize and to discharge the sensitiveness of the plain muscle of guinea-pigs, causing the anaphylactic type of response in the isolated plain muscle or shock following intravenous injection in sensitive animals.

METHODS.

Flukes were obtained from the livers of sheep killed on the same day. They were washed in at least four changes of sterile saline, slightly bruised either with forceps or in a mortar, and again washed in saline. After drying *in vacuo* over sulphuric acid they were powdered in a sterile agate mortar.

The possibility that extracts would be found to contain sheep serum protein, and even more than one antigenic helminthic substance, made the method of intravenous injection unsuitable for testing anaphylactic sensitiveness. The method of Dale and Laidlaw (1912) for testing the isolated uterus

of the virgin guinea-pig was therefore used. Certain modifications described in an earlier paper (Kellaway, 1928) enabled the partial pressure of CO_2 in the bicarbonate Ringer, and with it the P_H, to be kept moderately constant. The temperature was constant at 37·5°C. ± 0·1°C. The Ringer bath was of 50 c.cm. capacity, except where a larger bath is stated to have been used. The plain muscle was in no case perfused before testing. Guinea-pigs were sensitized by injecting them a few days after birth. They were allowed to remain with their mother for ten to fourteen days. Before being killed the external genitalia were inspected to avoid testing the uterus during oestrus (Stockard and Papanicolaou, 1919).

Extracts of fluke in sterile saline were made up immediately before use (0·1 gramme of dry powdered material to 20 c.cm. of saline). They were not filtered, and were kept in the cold, small samples being withdrawn as required for testing. They were used only in doses which had no stimulant effect on the normal uterus. In a few experiments strong extracts of undried flukes were used, and as these were found to be strongly stimulant to the isolated plain muscle of the normal guinea-pig, the solid matter was allowed to settle, and the supernatant fluid was diluted with equal bulk of sterile saline, and subjected to ultrafiltration of about 300 mm. Hg. pressure through pyroxylin membranes. After this treatment the extracts no longer had any stimulant action in a concentration of 1 in 25. The membranes were made at 20°C. by the method of Adair (1925). Five layers were poured at five minute intervals, and half an hour allowed for final drying before immersion in 70% alcohol for twelve hours. The membranes were impermeable to serum proteins and to starch, but permitted the passage of erythrodextrins.

Alcoholic extracts were made in absolute alcohol specially prepared by Mr. H. F. Holden (99·9 ± 0·1%), and in alcohol of lower strengths by volume made up from this. Alcoholic extracts were invariably filtered in the earlier experiments through Whatman No. 1 and in later experiments through Whatman No. 30 filter paper. The filtered extracts were then taken to dryness *in vacuo* at 35°C. to 40°C., and the residue was suspended in saline for use in sensitization and testing.

RESULTS.

(a) *Experiments in which Saline Extracts were used for Sensitization and Testing.*

These were five in number, and the results were regular. Female guinea-pigs were sensitized, three with 1·5 c.cm. of a fresh saline extract (1 fluke per c.cm.) and two with 1 c.cm. of saline extract, containing 5 mgm. of dried fluke per c.cm. Their plain muscle was tested between the 20th and 22nd days after injection, when the animals weighed from 210 to 260 grammes. In all cases

sheep serum was first added to the bath, and in one only was a trivial response obtained with a dose of 0·5 c.cm. Maximal anaphylactic reactions were then obtained with saline extract of fluke in doses of 0·1 c.cm. to 0·5 c.cm. In all the experiments but that figured (figure 2, iv) the repetition of the dose of saline extract caused no response. The reactions observed were therefore anaphylactic, and the example figured resembles the type of anaphylactic response not infrequently obtained with the plain muscle of guinea-pigs sensitized with hydatid scolices. These observations confirm those of Turner (1925), both in respect of the ease with which guinea-pigs may be sensitized with saline extracts and of the absence of sensitiveness to host protein.

In all these experiments care was taken to free saline extracts from pharmacological activity by dialysis or pressure filtration, or at least in the case of extracts freshly made up from dried material to use them in doses which were without effect on normal plain muscle. Whether this last precaution is sufficient is somewhat doubtful, since "sensitive" plain muscle may be more susceptible to the pharmacologically active substance present in fluke extracts than is the uterus of the normal guinea-pig. The reactions obtained were, however, definitely anaphylactic in type, and did not resemble the immediate histamine-like effect produced on normal uteri by fresh saline extracts of wet flukes.

The stimulant substance, if injected intravenously in sufficient dose, also kills guinea-pigs with symptoms resembling anaphylactic shock. A fresh extract of dried fluke, 0·1 in 10 c.cm. of saline, shaken for half an hour, and then cleared by centrifugation, was administered intravenously in a dose of 2·0 c.cm., and killed a normal guinea-pig weighing 165 grammes in 4 minutes. Smaller doses of 1·0 and 0·5 c.cm. produced no symptoms in other normal guinea-pigs. There are some indications that the pharmacologically active substance may be volatile, since on more than one occasion the ultrafiltrate from fluke extract, and the material in the filter when tested on normal plain muscle, both separately and together, were devoid of activity.

Despite the toxic action of fresh extracts, they may be used to produce sensitiveness, and this may be tested by the intravenous injection of doses too small to produce any symptoms in normal animals. Two guinea-pigs injected subcutaneously with 1·0 c.cm. of a fresh extract, were tested 28 days after sensitization. To avoid any possibility of host protein being responsible for the severe anaphylactic symptoms produced by the intravenous injection of saline extract of fluke, they had been injected subcutaneously 24 hours earlier with 0·1 c.cm. of sheep serum.

It seems fairly certain that the reactions observed with the plain muscle of animals sensitized with helminth extracts are not highly specific. The uteri of guinea-pigs sensitized with *Taenia saginata*, while they fail to react with ox serum, give with fluke extracts reactions indistinguishable from genuine

anaphylactic responses, and similar results are sometimes obtained with the plain muscle of guinea-pigs sensitized with saline scolex extract. Experiments in cross sensitization, and testing with *Taenia saginata* and hydatid scolex, have also been made, and will be recorded elsewhere. While no surprise can be occasioned by the crossed reactions which occur in this last case, the reaction of guinea-pigs sensitized with *Taenia saginata* or hydatid scolex to fluke extracts is somewhat unexpected.

(b) *Experiments in which Saline Extracts were used for Sensitization, and in which Extracts of Dried Fluke in Alcohol of various strengths were used for Testing.*

Four experiments of this kind were made. In two 1·5 c.cm. of fresh saline extract of fluke was used for sensitization, and on the 22nd day large doses of extract of 10% wet fluke in alcohol (final strength of alcohol 87·5% ± 0·2%) were used for testing without eliciting anaphylactic responses and without desensitizing the uterus, as was shown by the subsequent addition of saline fluke extract to the bath. In one experiment the residue from the evaporation of 48 c.cm. of alcoholic extract suspended in saline was used in divided doses (equivalent to 8, 16, 16, and 8 c.cm.) to treat one horn, and that from 30 c.cm. in divided doses (equivalent to 6, 12, and 12 c.cm.) to treat the other. After changing the Ringer both horns gave maximal reactions with saline extract in doses of 0·5 and 0·4 c.cm. in a bath of 120 c.cm. In another experiment a similar result was obtained after treating the uterus with the residue from the evaporation of 16 c.cm. of this alcoholic extract.

In a further experiment a guinea-pig, sensitized with 5 mgm. of dried fluke in 1 c.cm. of saline, was tested on the 21st day with extract of dried fluke in absolute, 80% and 60% alcohol. Figure 1 shows the result obtained. No reaction was observed after adding the residue from the evaporation of 10 c.cm. of 100% alcohol extract to the bath, and the residue from 5 c.cm. of 80% alcohol extract added next also gave no response. The residue of 5 c.cm. of 60% alcohol extract was now added, and gave a maximal anaphylactic reaction. Finally a small response was obtained with 0·5 c.cm. of saline fluke extract. With the second horn a maximal anaphylactic response was elicited with the residue from 5 c.cm. of the 80% alcohol extract, and following two such doses, the second of which produced no contraction, 0·5 c.cm. of saline fluke extract gave a maximal anaphylactic response. This experiment is not altogther in harmony with those recorded above, which suggest that extracts of fluke in 80% alcohol behave like the alcoholic tape-worm extracts described by Meyer (1911), being capable of sensitizing but not of causing anaphylactic shock. The absence of any evident desensitization following treatment with many successive doses of 80% alcohol extract is in strong contrast to the effect of a dose of 100% alcohol extract in desensitizing to the subsequent dose of 80% alcohol

extract, though not to that of the 60% extract. This last extract appears to be more effective in desensitizing against saline extract than is the same dose of 80% alcoholic extract of fluke.

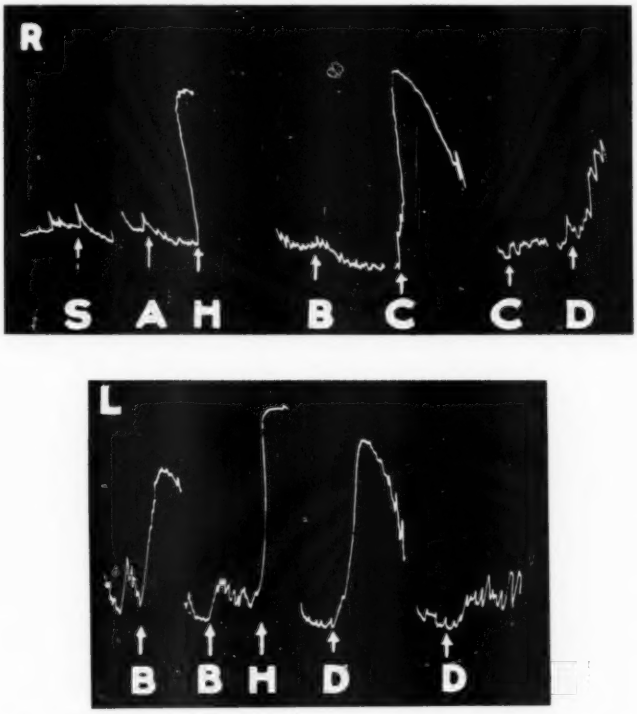


Figure 1. Responses of the right (R) and left (L) horns of the uterus of a guinea-pig weighing 259 grams sensitized by the injection 21 days earlier of 5 mgms. of dried fluke in 1·0 c.cm. of saline. At S 0·5 c.cm. of sheep serum; at A the residue from the evaporation of 10 c.cm. of a filtered extract of dried fluke (0·1 gram in 50 c.cm. of 100% alcohol); at B the residue from the evaporation of 5 c.cm. of a similar extract in 80% alcohol; at C the residue from the evaporation of 5 c.cm. of an extract in 60% alcohol; at D 0·5 c.cm. of a fresh dialyzed extract of fluke (0·2 gram in 50 c.cm.), and at H 0·002 mgm. of histamine added to the bath.

The 100% alcoholic extract contained only an extremely small amount of solid matter, and Mr. Holden found that the saline suspension of the residue of 25 c.cm. of this extract, and of that in 80% alcohol, taken up in 10 c.cm. of saline, gave negative Spiegler and haemochromogen reactions, whereas the residue of the 60% alcohol extract and the saline extract gave positive tests with both.

(c) *Experiments in which Alcoholic Extracts were used for Sensitization.*

The plain muscle of two guinea-pigs, sensitized with the residue from the evaporation of 8·0 c.cm. of alcoholic extract of wet flukes (87·5 ± 0·2% alcohol), gave maximal anaphylactic reactions when tested with doses ranging from 0·25 to 2·0 c.cm. of fresh dialyzed saline fluke extract in a bath of 120 c.cm. It was insensitive to sheep serum in a concentration of 1 in 60, and after three successive doses of the residue from the evaporation of 8 c.cm. of the alcoholic extract gave a definite anaphylactic response with 1·0 c.cm. of dialyzed saline fluke extract, a second dose causing no response. Four other guinea-pigs were sensitized with the residue from the evaporation of 25 c.cm. of an extract of 3·47 grammes of flukes (dried *in vacuo* over sulphuric acid at 7 mm. pressure) in 230 c.cm. of absolute alcohol. These were tested on the 22nd day, and were insensitive to sheep serum in a concentration of 1 in 50, to the residue from the evaporation of 20 c.cm. of the alcoholic extract of fluke, and with one exception to doses of 1 to 5·0 c.cm. of dialyzed saline fluke extract. In the single case in which sensitiveness to saline fluke extract was demonstrated the reactions in a bath of 120 c.cm. were as follows:

Right horn.	2·0 c.cm. Sheep serum.	Nil.
	Residue from 10 c.cm. of alcoholic extract.	Nil.
	3·0 c.cm. of saline extract.	Maximal response.
	3·0 c.cm. of saline extract.	Nil.
	0·01 mgm. of histamine.	Maximal response.
Left horn.	2·0 c.cm. of saline extract of fluke.	Maximal response.
	2·0 c.cm. of saline extract of fluke.	Nil.
	0·01 mgm. histamine.	Maximal response.

Two guinea-pigs were sensitized by the injection of the residue from the evaporation of 18 c.cm., and two with that from 9 c.cm. of an extract of dry flukes in absolute alcohol (0·5 gramme to 250 c.cm.). None of these were sensitive to sheep serum, and all gave maximal anaphylactic reactions with 0·2 to 0·5 c.cm. of fresh saline extracts when tested from the 18th to 21st day. Two of these experiments were specially planned to decide whether the 100% alcoholic extract of fluke could desensitize the uterus. The alcohol extract was filtered through No. 30 Whatman's filters to exclude the possibility of minute particles of suspended matter being carried through defective filter papers. Both these experiments clearly demonstrated the failure of these extracts to desensitize the sensitive plain muscle. In one the right horn gave a maximal anaphylactic response with 0·2 c.cm. saline extract, and the other horn behaved similarly after being treated with the residue from the evaporation of 45 c.cm. of absolute alcohol which had been shaken up in successive portions with 0·1 gramme of dry fluke. In the second experiment a horn treated with the residue from the evaporation of 150 c.cm. of alcohol used in successive fractions during the course of 48 hours at 15°C. to extract 0·1 gramme of dry fluke was not desensitized, subsequently giving a maximal reaction with 0·5 c.cm. of fresh

fluke extract. In the last case the residue from the evaporation of the alcoholic extract was added to the bath in successive portions without changing the Ringer, and was allowed to remain in contact with the plain muscle for half an hour.

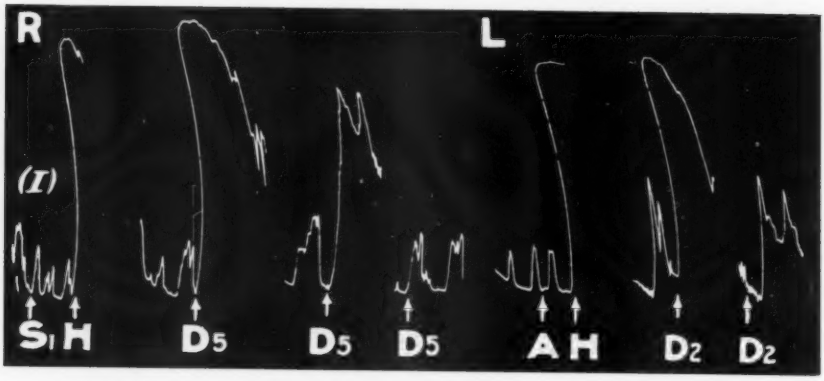
In a final series of experiments groups of guinea-pigs were sensitized with the residue from the evaporation of 250·0 c.cm. of extracts of fluke in 100%, 80%, and 60% alcohol by volume. The flukes were thoroughly washed, dried over sulphuric acid *in vacuo*, powdered, and again dried. Of this dry powder 0·5 grammes were shaken up at room temperature 15°C.–20°C. for five days with 250 c.cm. of each strength of alcohol in dry bottles with carefully ground stoppers. For use both for sensitization and testing the extracts were filtered through No. 1 Whatman papers before being taken to dryness *in vacuo* at 40°C. The plain muscle of guinea-pigs, sensitized with these alcoholic extracts, was tested on the 21st day, and the results are illustrated in figure 2 (i), (ii), and (iii). Of three animals which were sensitized with 100% alcohol extract, two were insensitive to sheep serum in a concentration of 1 in 100 and to 0·5 c.cm. of saline extract of fluke. The third animal gave the reactions recorded in figure 2 (i). It was insensitive to sheep serum and to the residue from 5·0 c.cm. 100% alcoholic extract of fluke, but gave a maximal anaphylactic reaction to 0·2 c.cm. of saline extract of fluke.

Of two guinea-pigs sensitized with 80% of alcoholic extract both were sensitive to 0·5 c.cm. of saline extract of fluke, giving maximal anaphylactic reactions. Figure 2 (ii) shows the reactions of one of these animals. The plain muscle gave a feeble anaphylactic response to the residue from the evaporation of 5 c.cm. of 80% alcoholic extract, and after this a maximal response to the residue from the evaporation of from 5 c.cm. of 60% alcoholic extract. This horn was then found to have become desensitized, a dose of 0·5 c.cm. of saline extract giving no response.

The results of sensitization with 60% alcohol extract are illustrated in figure 2 (iii). The plain muscle was insensitive to sheep serum, but reacted maximally to 1·0 c.cm. of saline extract of fluke. A small dose of this extract (0·02 c.cm.) produced only a very small effect on the other horn, and a subsequent dose of 0·1 c.cm. gave a rather more maximal response.

DISCUSSION.

The study of the antigenic properties of a helminth by the anaphylactic method is attended by various difficulties. It is not easy to obtain the substances to be tested in solution in saline. Alcoholic extracts, for example, have to be taken to dryness and re-suspended in saline or water, in which much of the alcohol-soluble matter is insoluble. If the isolated uterus be used for testing, the presence of more than one antigenically active substance appears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(I)
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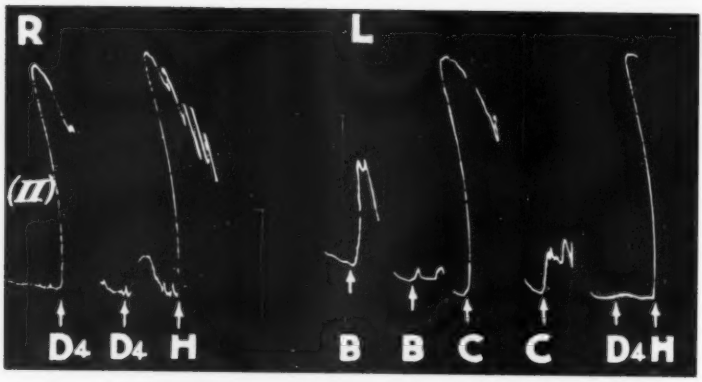
R
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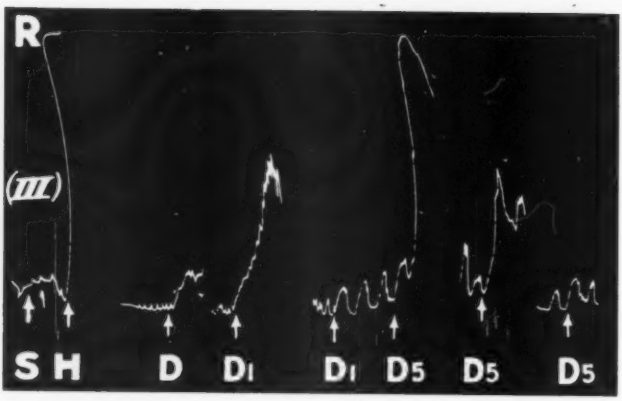
D4 H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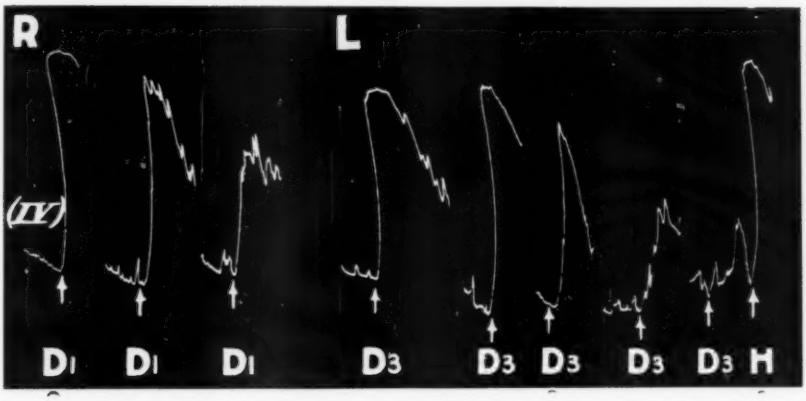

Figure 2. Responses of the right (R) and left (L) horns of the uteri of four guinea-pigs weighing from 220 to 270 grams sensitized by the injection 21 days earlier of (i) the residue from the evaporation of 25 c.cm. of a filtered extract of dried fluke (0·1 gram in 50 c.cm. of 100% alcohol), (ii) the residue from the evaporation of 25 c.cm. of a similar filtered extract in 80% alcohol, (iii) the residue from the evaporation of 25 c.cm. of a similar filtered extract in 60% alcohol, and (iv) 5 mgm. of dried fluke in 1·0 c.cm. of saline. At S 0·2 c.cm, at S_1 0·5 c.cm. of sheep serum; at A the residue from the evaporation of 5·0 c.cm. of 100% alcohol extract; at B that from 5·0 c.cm. of 80% extract; at C that from 5·0 c.cm. of 60% extract; at D 0·02 c.cm; D_1 0·1 c.cm., D_2 0·2 c.cm., D_3 0·25 c.cm., D_4 0·5 c.cm., D_5 1·0 c.cm. of fresh saline extract (equivalent to 0·1 gram in 25 c.cm.); at H 0·002 mgm. of histamine added to the bath.

to diminish the sensitiveness of the method, because the process of discharging the sensitiveness of the plain muscle to one antigen may impair its subsequent response to others. The time which is required for the development of sensitiveness may be different for the several antigenic substances present. Saline extracts of helminthic material frequently contain substances toxic for the guinea-pig, and possessing a stimulant action in the plain muscle. Finally the development of sensitiveness may be irregular, and this irregularity may depend either on the presence of bacteria in the extracts used for sensitization, saline extracts of parasitic material being rarely sterile, or on the fact that the sensitizing dose is near the limiting value for a particular animal.

While the results of sensitization with saline extracts of fluke appear to be quite regular, those with extracts made with absolute alcohol are somewhat irregular. If the anaphylactic antigen or antigens concerned be assumed to be of protein nature, this result might be anticipated, since the amount of protein present in extracts in 99·9% alcohol is too small to be detected by the most delicate chemical tests. It is not, however, safe to infer on chemical grounds that no helminthic protein is present, since guinea-pigs sensitized with extracts of carefully dried hydatid scolices from sheep cysts, in alcohol of similar strength, occasionally yield positive anaphylactic reactions with sheep

serum protein. The anaphylactic reaction far exceeds in sensitiveness any known chemical test for the presence of protein, and we cannot conclude that the anaphylactic reactions, which the plain muscle of guinea-pigs sensitized with alcoholic extracts of dry helminthic material exhibit somewhat irregularly when these are tested with saline extract, are certainly due to so-called "lipoid" antigens rather than to protein. The experiments with extracts in alcohol of varying strength clearly indicate their increasing potency in anaphylactic antigen, as the strength of the alcohol diminishes, and a similar effect has been demonstrated with alcoholic extract of hydatid scolices.

This evidence, if we were concerned with *one antigenic substance* only, would point to its being a protein, but it seems likely that in alcoholic extracts another substance is present which is antigenic only in a limited sense, behaving, like the tape-worm extracts described by Meyer (1911), and being capable only of sensitizing and not of causing shock. In support of this hypothesis is the fact that in no case in which sensitization has been produced with extracts of dried fluke in absolute alcohol have I been able to obtain definite anaphylactic reactions by testing with the suspended residue from the evaporation of such alcoholic extracts. This by itself, bearing in mind the greater sensitiveness of the reaction for sensitization than for testing might not be significant, but more significance attaches to the failure to desensitize the plain muscle by repeated large doses of the residue from the evaporation of filtered extracts of fluke in absolute alcohol.

There is no evidence that in complement fixation tests for helminths we are concerned with the same antigenic substances as those responsible for anaphylactic reactions, and Fairley's (1927) convincing experiments with cercarial alcoholic extracts show that not only are these capable of functioning as true antigens in complement fixation reactions, but that extracts in diminishing strengths of alcohol show corresponding diminution in effectiveness as antigens. Meyer's (1910-12) earlier experiments with watery and alcoholic extracts of tape-worm led him to the conclusion that the antigenic substance was of "lipoid" nature akin to lecithin, and Fairley holds a somewhat similar view. Turner (1926), using Fairley's alcoholic extracts of cercariae, was unable to sensitize guinea-pigs with them, but does not appear to have used saline extracts for testing. For this reason his observations cannot be held to exclude the presence of traces of protein in these alcoholic extracts, nor of a substance capable of sensitizing, but not of causing anaphylaxis. Quantitative considerations, however, make it almost certain that the "antigen" responsible for complement fixation with this helminth is not a protein. In complement fixation we are concerned with the study of the reaction produced, not with large amounts of alcoholic extracts, like those used for sensitization and testing in the present experiments, but with minute amounts (the extract being considerably diluted, so that even if traces of protein are present in

them, it is probable that they owe their activity as antigens in these *in vitro* tests to substances relatively soluble in alcohol, and akin to the lipins rather than to the proteins.

On the other hand, the evidence presented here in regard to fluke and similar unpublished experiments with hydatid scolex extracts suggest that the saline soluble anaphylactic antigens may be proteins. The fact that with both fluke and scolex alcoholic extracts decreasing strengths of alcohol used for extraction appear to afford increasingly strong solutions of anaphylactic antigen favours this view, though the resistance of these antigens to heat suggests that they must be very different in nature from other proteins.

The substances that are present in absolute alcohol extracts of fluke, and which give rise to anaphylactic sensitiveness, may be more nearly akin to the complement fixing antigens, and may owe their activity to union with protein in the body of the guinea-pig, thus producing a *foreign* complex to which the animal can produce antibody.

CONCLUSIONS.

There appear to be two antigenic substances in extracts of fluke, one present in saline extracts, which though heat stable shows a decreasing solubility in increasing strengths of alcohol, and is a true anaphylactic antigen, and another, probably a lipin, soluble in absolute alcohol, and capable of sensitizing guinea-pigs, but not of discharging the sensitiveness of the sensitive plain muscle. The latter substance may possibly be identical with the antigen responsible for complement fixation.

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ANAPHYLACTIC EXPERIMENTS WITH GLOBIN

by

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Much work has been done to decide whether oxyhaemoglobin is antigenic. Hektoen and Schulhof (6) produced precipitating antisera in rabbits by the injection of about 0·15 grm. of uncrystallized ox oxyhaemoglobin into each rabbit. They have (7) more recently found that sheep globin reacts with a precipitating antiserum to sheep oxyhaemoglobin. Schmidt and Bennett (10), using crystallized dog oxyhaemoglobin, injected 0·6–0·8 grm. into each rabbit. They obtained no precipitating or complement-fixing antibody in the sera of such injected rabbits, and were unable to produce anaphylactic sensitiveness in guinea-pigs. Heidelberger and Landsteiner (5) found that a series of injections, totalling about 9–10 grm. of very carefully recrystallized horse oxyhaemoglobin per rabbit, only yielded good antisera in a small proportion of their animals. In this connection reference may be made to the work of Browning and Wilson (2), of Schmidt (9), and to the classical researches of Gay and Robertson (4). In these experiments, however, denatured globin was used, which differs widely from globin in its chemical and physical properties. Again, according to Bruynoghe (3), denaturation of a protein renders it antigenically distinct from the original native protein. This distinction is also discussed by Hektoen and Schulhof (7). The results of many other workers are reviewed in the papers referred to.

This paper records the results of some experiments on globin as prepared by Hill and Holden (8). In order to obtain more concentrated solutions of globin it was dialyzed under pressure against distilled water at 0°C. Before use sodium chloride was added to make the solution 0·9%. Some initial experiments were made with ox globin, but it was abandoned in favour of horse globin, as some guinea-pigs sensitized with the former reacted fatally to an intravenous injection of ox serum, in spite of repeated and careful washing of the corpuscles. The stroma protein used in some of the experiments was made from thoroughly washed horse corpuscles. The corpuscles were laked with two volumes of water, and enough sodium chloride was added to make the solution 2%. The stroma was thrown down by centrifuging, and washed with 2% sodium

chloride solution until the spectrum of oxyhaemoglobin could no longer be seen. The stroma protein was washed with distilled water, alcohol, and ether, and kept in a dry state. Before use it was dissolved in 0·3 N sodium hydroxide, and the solution immediately neutralized with 0·3 N hydrochloric acid, thus yielding a fine suspension of stroma protein in normal saline.

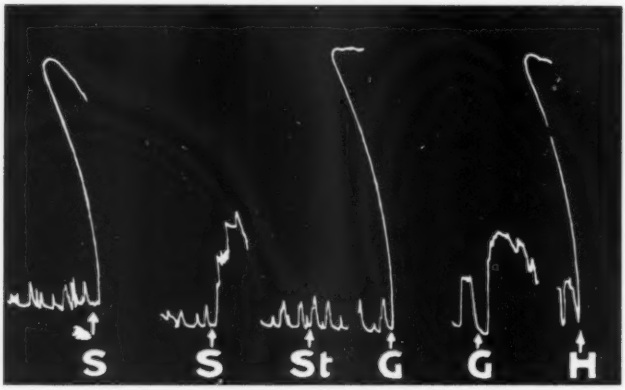

Figure 1. Reactions of the right horn of the uterus of a guinea-pig sensitized 25 days previously by the injection of 40 mgm. of horse globin. At S. 0·5 c.cm. of horse serum, at St. 10 mgm. of horse stroma protein, at G. 5 mgm. of horse globin, and at H. 0·002 mgm. of histamine added to the bath.

The injections and the experiments with the isolated uterus were kindly performed for me by Dr. C. H. Kellaway. In the latter the technique of Dale and Laidlaw was followed, and the uterus was suspended in a bath of 50 cc. capacity. The guinea-pigs received the first subcutaneous injection when about a week old, and weighing less than 100 grm. They were left with their mothers, and grew rapidly, gaining about 8 grm. per day in weight. The results of the tests are given in Tables 1 and 2, and the figure is a tracing of guinea-pig 3 in Table 2.

TABLE 1.

Results of Intravenous Injections in Young Guinea-pigs Sensitized by a Subcutaneous Injection of Horse Globin.

Weight in gms. when tested.	Sex.	Sensitizing dose, mgm. of globin.	Number of days sensitized.	Testing dose.	Results.
270	F	9	22	9 mgm. globin	No symptoms.
230	F	9	22	18 mgm. globin	No symptoms.
245	F	9	22	18 mgm. globin	No symptoms.
260	F	9	22	1 cc. horse serum	No symptoms.
282	F	9	27	29 mgm. globin	No symptoms.

Weight in gms. when tested.	Sex.	Sensitizing dose, mgm. of globin.	Number of days sensitized.	Testing dose.	Results.
263	F	9	27	29 mgm. globin	No symptoms.
247	F	9	27	33 mgm. globin	No symptoms.
322	M	45	27	40 mgm. globin	No symptoms.
249	M	45	27	40 mgm. globin	No symptoms.
250	M	45	27	40 mgm. globin	No symptoms.
326	M	45	27	40 mgm. globin	Died in 3½ minutes. Symptoms not typically anaphylactic, but Auer and Lewis phenomenon at autopsy.
291	M	45	27	40 mgm. globin	No symptoms.
305	M	45	27	40 mgm. globin	No symptoms.
275	M	45	27	0.5 cc. horse serum	Slight tremors, recovered in 10 minutes.
Same animal 30 minutes later.				40 mgm. globin	Slight collapse, recovered in 10 minutes.
300	M	45	27	40 mgm. globin	No symptoms.
305	M	45	27	0.5 cc. horse serum	No symptoms.
Same animal 10 minutes later.				40 mgm. globin	No symptoms.

TABLE 2.

Results of Tests on the Isolated Uteri of Young Guinea-pigs Sensitized with Horse Globin.

Weight when tested.	Sensitizing dose.	No. of days sensitized.	Testing dose.	Response of plain muscle.
242 gm.	9 mgm. globin	27	1 cc. horse serum	Nil
			15 mgm. globin	Maximal
			15 mgm. globin	Nil
			0.002 mgm. histamine	Maximal
240 gm.	40 mgm. globin	22	0.2 cc. horse serum	Delayed maximal
			0.2 cc. horse serum	Small
			2 mgm. horse stroma protein	Nil
			10 mgm. horse globin	Maximal
			10 mgm. horse globin	Moderate
			10 mgm. horse globin	Small
			0.002 mgm. histamine	Maximal
260 gm. (See figure)	40 mgm. globin	25	0.5 cc. horse serum	Maximal
			0.5 cc. horse serum	Small
			10 mgm. horse stroma protein	Nil
			5 mgm. horse globin	Maximal
			5 mgm. horse globin	Small
			0.002 mgm. histamine	Maximal

These results may be summarized as follows. Of fifteen animals injected intravenously one died, with symptoms which were not typically anaphylactic, though at autopsy the lungs were found to be distended, as described by Auer and Lewis (1). One other animal showed slight collapse. Only one of three animals injected with horse serum showed any symptoms, and these were only slight. The plain muscle of one was insensitive to horse serum protein; that of the others was definitely sensitive to it in a moderately high concentration. After desensitizing with horse serum all the uteri were sensitive to some substance in the globin solution, which was without effect on a normal uterus in

the concentration used. In order to obtain the positive results in Table 2 much larger amounts of protein were required for sensitization than would have been needed for sensitization with the serum protein. In view of the practical impossibility of being certain that corpuscles do not contain small amounts of an antigen of a potency comparable with that of serum protein, it seems probable that the biologically specific substance in the red blood corpuscles is other than oxyhaemoglobin. This view is rendered probable by the observation of Hektoen and Schulhof (6), that by acidification and subsequent neutralization the whole of the oxyhaemoglobin can be removed from an extract of corpuscles without seriously impairing the antigenic power of the remaining fluid. Should such a substance be present, and its relationship with the blood pigment be that of adsorption, no reasonable number of recrystallizations of the latter could be expected to free it adequately from the former.

SUMMARY.

The injection of horse globin does not induce fatal anaphylaxis in guinea-pigs.

Experiments with the isolated uterus suggest that it becomes sensitive to a protein in the globin solution, which is neither stroma nor serum protein. Quantitative considerations render it unlikely that it is globin.

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